



## Discovery and molecular biology of the abortive infection phage resistance system AbiV from *Lactococcus lactis*

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*Publication date:*  
2008

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*  
Haaber, J. B. B. (2008). *Discovery and molecular biology of the abortive infection phage resistance system AbiV from Lactococcus lactis*.

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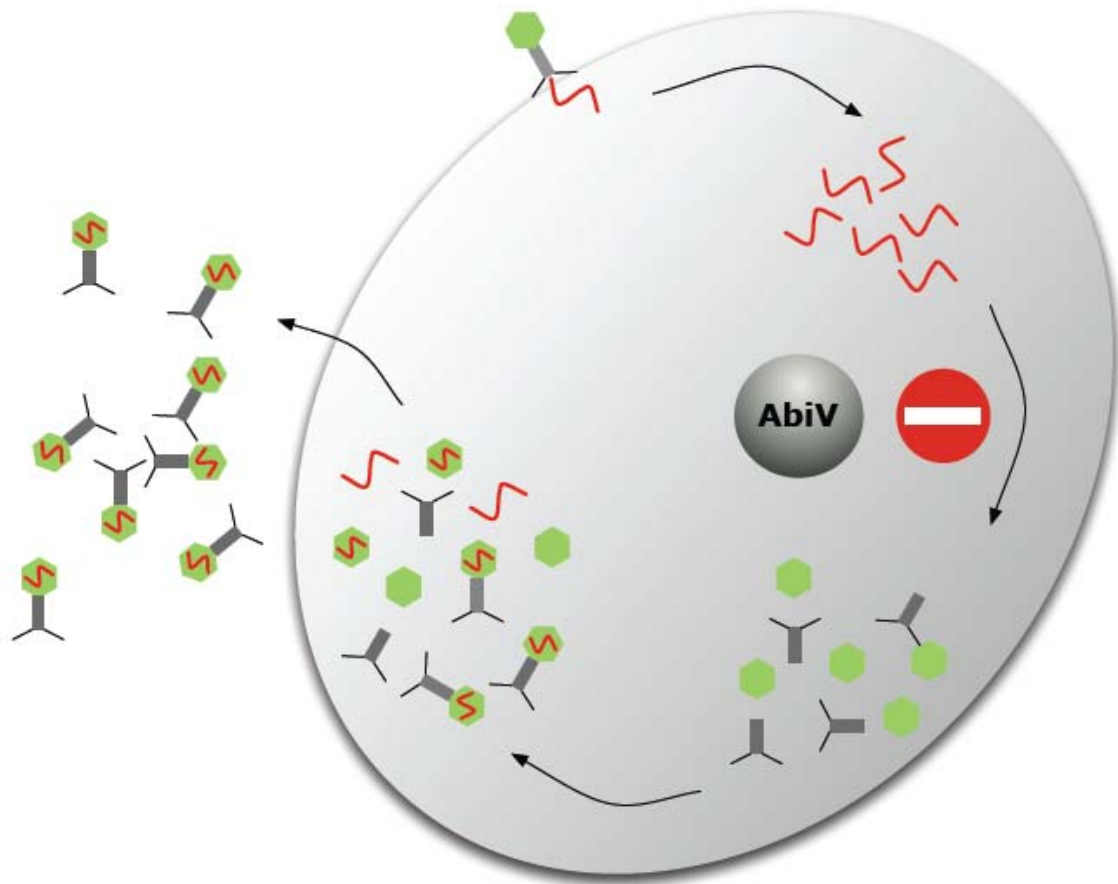
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# Discovery and molecular biology of the abortive infection phage resistance system *AbiV* from *Lactococcus lactis*

Jakob Haaber



Ph.D. thesis, September 2008



Discovery and molecular biology of the abortive  
infection phage resistance system AbiV from  
*Lactococcus lactis*

Ph.D. Thesis

Jakob Haaber

September 2008

Department of Systems Biology  
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Denmark



## **Preface**

The present thesis is submitted in candidacy for the PhD degree from the Technical University of Denmark (DTU). It contains the results obtained during my pre-doctoral work from March 2005 to September 2008 which was mainly carried out at Center for Systems Microbiology, Department of Systems Biology, DTU but also during a 5 month stay at Université Laval, Quebec, Canada.

The study was funded by DTU as a PhD scholarship. Additional funding from the Ministry of Science (administered by the Joint Proof of Concept Consortium) permitted a 7 month project for developing the commercial value of an invention which was made during the PhD study and which is now being patented. This study was supervised by Professor Karin Hammer, Department of Systems Biology, DTU assisted by Professor Sylvain Moineau, Département de biochimie et de microbiologie, Université Laval, Quebec, Canada.

I sincerely thank my supervisor Karin Hammer for accepting me in her group despite my initial limited knowledge of molecular biology and for believing in me throughout the project. Without her constantly inspiring guidance and the endless valuable discussions this work could not have been completed.

A special thanks to Sylvain Moineau for his excellent guidance and encouragement as well as his great hospitality which made my stay in Quebec an unforgettable experience.

I thank my colleagues at Center for Systems Microbiology and the people in the lab of Sylvain Moineau for creating an every-day pleasant working environment.

Finally, I thank my friends and family and especially Axelle for her loving support and encouragement, and for understanding that the investigation of two proteins can be very important.

Kgs. Lyngby, 15. September 2008

Jakob Haaber



# Contents

Abstract.....	2
Dansk resumé.....	4
List of publications.....	6
Co-author statements.....	7
Aim and outline of thesis.....	12
<b>CHAPTER 1: INTRODUCTION.....</b>	<b>15</b>
<i>Lactococcus lactis</i> .....	17
Lactococcal phages.....	17
Phage sk1 as a representative of 936-species.....	18
Phage biology.....	20
Natural phage resistance mechanisms.....	24
<i>E. coli</i> phage exclusion mechanisms.....	37
Other phage resistance mechanisms.....	38
<b>CHAPTER 2: MANUSCRIPT I.....</b>	<b>41</b>
AbiV, a novel abortive phage infection mechanism on the chromosome of <i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363.....	43
<b>CHAPTER 3: MANUSCRIPT II.....</b>	<b>71</b>
Identification and characterization of a phage gene <i>sav</i> involved in sensitivity to the lactococcal abortive infection mechanism AbiV.....	73
<b>CHAPTER 4: MANUSCRIPT III.....</b>	<b>99</b>
Direct interaction between the lactococcal abortive infection protein AbiV and the phage protein SaV prevents translation of phage proteins....	101
<b>CHAPTER 5: MANUSCRIPT IV &amp; V.....</b>	<b>127</b>
Industrial applications of the novel lactococcal abortive infection mechanism AbiV.....	129
PCT patent application.....	140
<b>CHAPTER 6 DISCUSSION AND OUTLOOK.....</b>	<b>195</b>
Description of the AbiV phage resistance system.....	197
Perspectives and outlook.....	205
<b>REFERENCES.....</b>	<b>209</b>



## Abstract

Lactic acid bacteria (LAB) are used in the production of fermented food products but bacteriophage (phage) killing of susceptible cultures is a persistent problem for the use of LAB in industrial fermentations. Through the time of evolution a number of different phage resistance mechanisms have evolved to protect the bacteria by attacking different points in the life cycle of infecting phages. One group of phage resistance mechanisms is called abortive infection (Abi), which functions after phage DNA has been transferred to the infected cell but before cell lysis. Through the molecular actions of a given Abi system, phage proliferation is halted and the infected cell dies. Due to the commercial interest in protecting LAB against phage attacks, the Abi systems of especially *Lactococcus lactis* have been studied intensively during the last decades and to date twenty-three natural Abi systems have been isolated from this species. However, except for a few cases, the detailed molecular modes of action (MOA) of these systems remain largely unknown.

We discovered the new phage resistance mechanism AbiV on the chromosome of *L. lactis* subsp. *cremoris* MG1363 using insertional mutagenesis in which the silent *abiV* gene was turned on by a promoter in a plasmid integrated on the chromosome. AbiV provides protection against phages from the 936 and c2 species with an efficiency of plaquing (EOP) of ca  $10^{-4}$  but it is not active against the P335 species. The AbiV system consists of a single gene with low G+C content that encodes a protein of 201 amino acids with no similarity to other known proteins. Native AbiV forms a dimer which is non-toxic and most likely exists in the cytosol of the cell.

AbiV insensitive phage mutants were isolated and analyzed to elucidate factors involved in sensitivity of the wild type phages to AbiV. Whole genome sequencing was carried out for the phage mutant p2.1 (936 species) while selected regions of the genomes were sequenced in the 936-like phage mutants sk1.1, jj50.1, P008.1, bIL170.1 and the c2-like phage mutants c2.1 and bIL67.1. Sequence analysis revealed mutations in the early transcribed gene *sav* which was concluded to be responsible for the AbiV sensitive phenotype. The translated SaV protein consists of 128 amino acids and no putative function could be assigned based on database sequence similarity searches. The protein forms a dimer, which exhibits a strong and fast working toxic effect in both *Escherichia coli* and *L. lactis* when overexpressed. However, this effect is relieved when a mutated version of the protein is cloned and expressed. The majority of the mutations discovered in the phage mutants

effectively prevent translation of the SaV protein (nonsense mutations, deletion of start codon). Interestingly, most mutations were located in a region of the protein which is conserved among a wide array of phage species and a phylogenetic analysis of this region suggests that it has evolved from a common ancestor. We suggest that the conserved region is involved in sensitivity to AbiV and that it might also be responsible for the antimicrobial activity of the native SaV protein.

Using several independent methods, AbiV and SaV were demonstrated to interact directly most probably as two homodimers (AbiV<sub>2</sub>SaV<sub>2</sub>). The effects of this interaction on the lytic cycle of phage p2 (936-species) were investigated and we found that while RNA synthesis was only slightly affected, protein synthesis was severely inhibited shortly after phage infection. More details were revealed using mRNA dot blot and western blotting. Phage gene transcription was shown to be more inhibited at later stages in the phage lytic cycle, while translation of both early and late phage proteins was completely inhibited shortly after infection. It was therefore concluded that the AbiV system functions by inhibition of the translation apparatus of the cell. The phenotypic effects of the AbiV system on phage development were different between phages from the 936 species and c2 species. In the former, DNA replication occurred and concatemeric DNA was accumulated, whereas in the latter, no DNA replication was observed. Furthermore, to be efficient against c2 phages a higher expression of AbiV was needed compared to what was required to provide protection against 936 phages. Assuming that the AbiV system inhibits the cellular translation apparatus, we propose a model to explain that the different phenotypic observations can be caused by the same molecular MOA. This includes SaV activation of the AbiV protein which might undergo conformational changes thereby exposing a putative RNA binding site.

Finally, the industrial applications of the AbiV system were investigated by the isolation of bacterial mutants spontaneously expressing AbiV to gain phage resistance. It was further demonstrated that *abiV* can be transferred between different bacteria using chromosomal conjugation. To the best of our knowledge it is the first time that conjugal transfer of a chromosomally encoded phage resistance mechanism has been demonstrated.

## Dansk resumé

Mælkesyrebakterier bruges til produktion af fermenterede fødevarer men bakteriofag (fag) angreb af sensitive kulturer er et stort og vedvarende problem for den industrielle brug af mælkesyrebakterier til fermentering. I løbet af evolutionen er der blevet udviklet en række forskellige typer fag-resistens mekanismer der beskytter bakteriecellen ved at angribe inficerende fager forskellige steder i deres livscyklus. En gruppe af fag-resistens mekanismer hedder abortiv infektion (Abi), og de virker ved at hæmme faktorer i fagens lytiske cyklus efter at DNA er blevet overført til den inficerede celle men før celle lysis. Som følge af de molekylære mekanismer for et givent Abi system bliver udviklingen af nye fag partikler standset og den inficerede celle dør. På grund af den kommercielle interesse i at beskytte mælkesyrebakterier mod fag-angreb, er Abi systemer fra specielt *Lactococcus lactis* blevet undersøgt grundigt hvilket har resulteret i isolering af 23 naturlige Abi systemer fra denne art. Imidlertid er de specifikke molekylære mekanismer der fører til Abi fænotypen stort set ukendte når man ser bort fra enkelte velundersøgte Abi systemer.

Vi identificerede den nye fag resistens mekanisme AbiV på kromosomet af *L. lactis* subsp. *cremoris* MG1363 ved hjælp af mutagenese med et insertion element lokaliseret på et plasmid, hvilket førte til at *abiV* der normalt ikke udtrykkes i denne bakterie blev udtrykt fra en promoter i det kromosomalt integrerede plasmid. AbiV beskytter mod infektion af fager fra arterne 936 og c2 med en plaquing effektivitet (EOP) på ca.  $10^{-4}$ , men virker ikke mod P335 fager. AbiV systemet består af et enkelt gen med lavt G+C indhold som koder for et protein med 201 aminosyrer der ikke har signifikant identitet til andre kendte proteiner. Det native AbiV protein danner en dimer som ikke er giftig for bakteriecellen og som sandsynligvis eksisterer i cellens cytosol.

Fag mutanter som ikke er sensitive til AbiV blev isoleret og analyseret for at belyse hvilke faktorer der er involveret i vild-type fagernes sensitivitet til AbiV. Vi sekventerede det komplette genom fra fag mutanten p2.1 (fra arten 936) foruden udvalgte regioner på genomet af 936-fag mutanterne sk1.1, jj50.1, P008.1, bIL170.1 samt c2-fag mutanterne c2.1 og bIL67.1. Sekvens analyser viste at mutationerne forekom i det tidligt transskriberede fag gen *sav* og vi konkluderede derfor at dette gen er ansvarligt for fagens sensitivitet til AbiV. Det udtrykte SaV protein består af 128 aminosyrer og det var ikke muligt på baggrund af database søgninger at knytte en formodet funktion til proteinet. Det native protein danner en dimer som har en stærk og hurtigtvirkende toksisk effekt

både når det bliver overudtrykt i *Escherichia coli* og *L. lactis*. Denne toksiske effekt ses dog ikke når en muteret udgave af proteinet bliver klonet og udtrykt. De fleste mutationer der blev opdaget i de sekventerede fag-mutanter forhindrer translationen af det oprindelige SaV protein (pga. nonsens mutationer og deletion af start-kodon). Det er interessant at bemærke at de fleste mutationer ligger i en del af proteinet som er meget konserveret i en lang række af fag-arter og en fylogenetisk undersøgelse af denne region antyder desuden en evolutionær sammenhæng mellem de forskellige fag-proteiner. Vi foreslår at den konserverede del af SaV er involveret i fagens sensitivitet til AbiV og at den muligvis også er ansvarlig for den antimikrobielle funktion i SaV proteinet.

Adskillige metoder blev brugt til at vise at AbiV og SaV interagerer direkte samt at dette sandsynligvis sker mellem to homodimerer (AbiV<sub>2</sub>SaV<sub>2</sub>). Effekten af denne interaktion på den lytiske cyklus i fagen p2 (fra arten 936) blev undersøgt og vi fandt ud af at mens RNA biosyntesen kun blev påvirket minimalt blev protein biosyntesen hæmmet betragteligt kort efter faginfektionens start. Et mere detaljeret billede fremkom ved at bruge mRNA dotblot samt western blotting. Her blev det vist at fagens gen transskription var kraftigere nedsat jo senere i den lytiske cyklus genet blev transskriberet mens translationen af både tidlige og sene fagproteiner var fuldstændig standset kort tid efter infektionens start. Det blev derfor konkluderet at AbiV systemet virker ved at hæmme translations-apparatet i den inficerede celle. AbiV systemets fænotypiske effekter på den lytiske cyklus var forskellig fra 936 fager til c2 fager. Således observerede vi DNA replikation i 936 fager der resulterede i akkumulering af konkateneret fag DNA hvorimod DNA replikation ikke blev observeret i c2 fager. Derudover var et højere niveau af AbiV nødvendigt for at beskytte bakterien mod infektion af c2 fager i forhold til infektion af 936 fager. Vi antager at AbiV systemet virker ved en effektiv hæmning af cellens translations apparat og vi fremsætter desuden en model der skal forklare at de forskellige fænotypiske observationer kan forårsages af den samme underliggende molekulære mekanisme. Modellen inkluderer at SaV proteinet aktiverer AbiV proteinet som derved muligvis ændrer konformation således at et formodet RNA bindende område bliver eksponeret.

Endelig blev de industrielle anvendelsesmuligheder af AbiV systemet undersøgt. Vi viste af bakterien kan mutere til spontant at udtrykke AbiV hvorved den opnår fagresistens. Endvidere blev det demonstreret at *abiV* genet kan flyttes mellem forskellige bakterier ved at benytte kromosomal konjugation. Det er, så vidt vides, første gang det er blevet vist at en kromosomal fag-resistens mekanisme kan overføres mellem bakterier ved hjælp af konjugation.

## List of publications

The scientific results of this thesis have resulted in the following manuscripts:

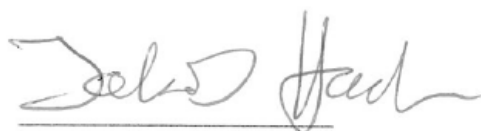
- I. **Haaber, J., Moineau, S., Fortier, L-C., Hammer, K.** (2008). AbiV, a novel abortive phage infection mechanism on the chromosome of *Lactococcus lactis* subsp. *cremoris* MG1363. *Applied and Environmental Microbiology* (accepted for publication).
- II. **Haaber, J., Hammer, K., Moineau, S.** (2008). Identification and characterization of a phage gene *sav* involved in sensitivity to the lactococcal abortive infection mechanism AbiV. *Applied and Environmental Microbiology* (submitted).
- III. **Haaber, J., Labrie, S., Cambillau, C., Moineau, S., Hammer, K.** (2008). Direct interaction between the lactococcal abortive infection protein AbiV and the phage protein SaV prevents translation of phage proteins. (Manuscript)
- IV. **Haaber, J., Moineau, S., Hammer, K.** (2008). Industrial applications of the novel lactococcal abortive infection mechanism AbiV. (Manuscript)
- V. **Haaber, J., Moineau, S., Hammer, K.** (2008). Phage resistance. International PCT patent application, filed to the Danish Patent and Trademark Office.

## Co-author statements

### Paper I

Haaber, Jakob; Moineau, Sylvain; Fortier, Louis-Charles; and Hammer, Karin (2008). AbiV, a novel abortive phage infection mechanism on the chromosome of *Lactococcus lactis* subsp. *cremoris* MG1363. *Applied and Environmental Microbiology* (in press).

All experiments were carried out by JH except construction of the pLC5 vector which was done by LCF. Data analysis as well as writing and submission of the manuscript were done by JH supervised by KH and SM.



Jakob Haaber



Louis-Charles Fortier



Sylvain Moineau



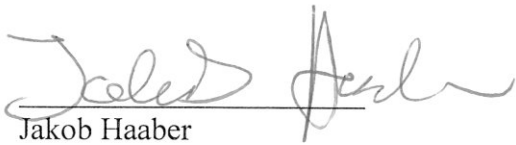
Karin Hammer

## Paper II

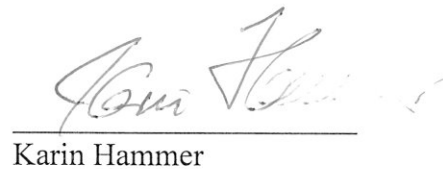
Haaber, Jakob; Hammer, Karin; and Moineau, Sylvain (2008). Identification and characterization of a phage gene *sav* involved in sensitivity to the lactococcal abortive infection mechanism AbiV.

*Applied and Environmental Microbiology* (submitted)

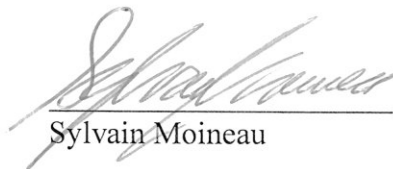
All experiments were carried out by JH. whole genome sequencing was planned and prepared by JH supervised by SM. Data analysis as well as writing and submission of the manuscript was done by JH supervised by KH and SM.



Jakob Haaber



Karin Hammer



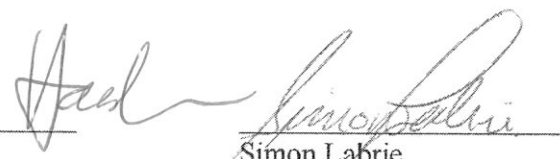
Sylvain Moineau

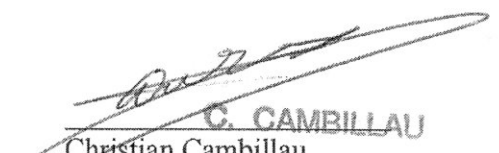
### Paper III

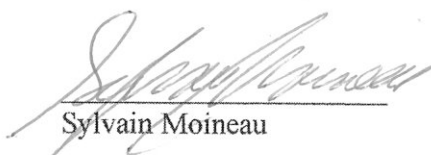
Haaber, Jakob; Moineau, Sylvain; Labrie, Simon, Cambillau, Christian; and Hammer, Karin (2008). Direct interaction between the lactococcal abortive infection protein AbiV and the phage protein SaV prevents translation of phage proteins. (*Manuscript*).

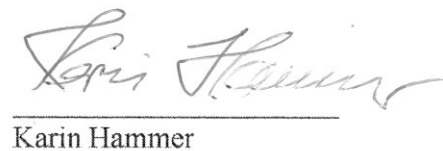
All experiments were carried out by JH except the protein interaction experiment using WYATT and Fluorescence, which was performed by SL. SL also analyzed the data and wrote the part of the manuscript dealing with the WYATT and fluorescence experiment. All other data analysis as well as writing of the manuscript were done by JH supervised by KH, SM and CC.

  
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Karin Hammer



#### **Paper IV**

Haaber, Jakob; Moineau, Sylvain; and Hammer, Karin (2008). Industrial applications of the novel lactococcal abortive infection mechanism AbiV. *Manuscript*.

All experiments were carried out by JH. Data analysis as well as writing and submission of the manuscript was done by JH supervised by KH and SM.




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Jakob Haaber



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Sylvain Moineau



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Karin Hammer

## Paper V

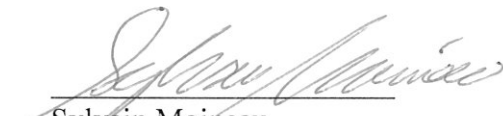
Haaber, Jakob; Moineau, Sylvain; and Hammer, Karin (2008). Phage resistance. International PCT patent application, filed to the Danish Patent and Trademark Office.

All experiments were carried out by JH except construction of the pLC5 vector which was done by LCF (as for paper I). Data analysis was done by JH supervised by KH and SM. Writing of the application was done by JH assisted by the patenting bureau Plougmann & Vingtoft (PV) and supervised by KH and SM. Filing was done by PV.



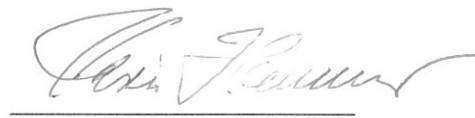
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Jakob Haaber



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Sylvain Moineau



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Karin Hammer

## Aim and outline of thesis

The aim of this PhD study was to identify factors involved in phage resistance in the lactococcal model bacterium *L. lactis* subsp. *cremoris* MG1363 and subsequently reveal the molecular mechanisms leading to the phage resistance phenotype. Upon identification of the phage resistance mechanism AbiV, the focus was to reveal which factors in an infecting phage that triggers the Abi phenotype as well as the kind of interaction between the Abi mechanism and the phage lytic cycle. Investigations were carried out mainly using 936 phages due to the abundance of information available on these phages. The industrial applicability of the AbiV system was also investigated as part of a 7-month patent proof-of-concept project.

A general introduction to phage biology, lactococcal phages, and phage resistance mechanisms with emphasis on Abi mechanisms is presented in Chapter 1. The following chapters (2-5) contain the obtained results which are presented in 5 individual manuscripts. Chapter 2 contains the manuscript: “Identification of AbiV, a novel abortive phage infection mechanism on the chromosome of *L. lactis* subsp. *cremoris* MG1363” (Manuscript I), which describes the discovery and microbiological characterization of AbiV. This manuscript has been accepted for publication in Applied and Environmental Microbiology (AEM). Manuscript II (Chapter 3) which is named: “Identification and characterization of a phage gene *sav* involved in sensitivity to the lactococcal abortive infection mechanism AbiV”, describes the identification of SaV which is involved in AbiV sensitivity and exhibits a strong antimicrobial effect in *L. lactis* and *E. coli*. This manuscript was submitted to AEM prior to submission of the present thesis. Chapter 4 consists of the manuscript entitled: “Direct interaction between the lactococcal abortive infection protein AbiV and the phage protein SaV prevents translation of phage proteins” (Manuscript III). In this manuscript we provide evidence for the direct protein interaction between AbiV and SaV and we describe the molecular mode of action of the system which inhibits the cellular translational apparatus. This manuscript awaits ongoing work focused on resolving the three dimensional structure of AbiV before submission to a hitherto not specified journal. Chapter 5 contains 2 manuscripts. The first (Manuscript IV) is entitled: “Industrial applications of the novel lactococcal abortive infection mechanism AbiV” and it demonstrates spontaneous expression and conjugal transfer of AbiV. This manuscript will be submitted as a note to AEM shortly after submission of this thesis. The second manuscript (Manuscript V) is an international PCT patent application covering the phage resistance

properties of AbiV and SaV. The application was filed to the Danish Patent and Trademark Office in July 2008. Chapter 6 contains a summary and general discussion of the obtained results along with suggestions for future work. References are listed individually for each manuscript and references outside the manuscripts are found at the end of the thesis.



# **INTRODUCTION**



# INTRODUCTION

## *LACTOCOCCUS LACTIS*

The Gram-positive bacterium *Lactococcus* belongs to the heterogeneous group of lactic acid bacteria (LAB) with a low genomic G-C content. Lactococci are non-sporulating, aerotolerant and acid-tolerant. They lack a respiratory chain and supply their energy by means of fermentation in which they convert carbohydrates into the main end product, lactic acid <sup>146</sup>. *L. lactis* is used as a starter culture in industrial fermentations for cheese production and the world-wide use of this bacterium produces annually an estimated 10<sup>7</sup> tonnes of cheese, which makes lactococcal milk fermentation the largest volume of bacterial culture controlled by man <sup>26,57</sup>. A typical mixed starter culture consists of 2-3 well defined strains and the activity and specific properties of these strains are important for the consistency of the end product quality. A major problem for industrial milk fermentations is the aseptic nature of the fermentation process, which makes the starter cultures vulnerable to attack by ubiquitous phages. Since the discovery of phages in milk fermentations in 1935 <sup>155</sup>, dairy scientist have put a great effort into preventing phage mediated fermentation failures. Still, 70 years later, phages remain a significant problem for the dairy industry and it is estimated that ca 0.1% to 10% of all milk fermentations are negatively affected by phage attacks <sup>109</sup>. This has serious economic consequences for the dairy industry which is dependent on specific quality requirements of their products and therefore significant resources have been spent on research that can protect the vulnerable production strains against phage attack.

## LACTOCOCCAL PHAGES

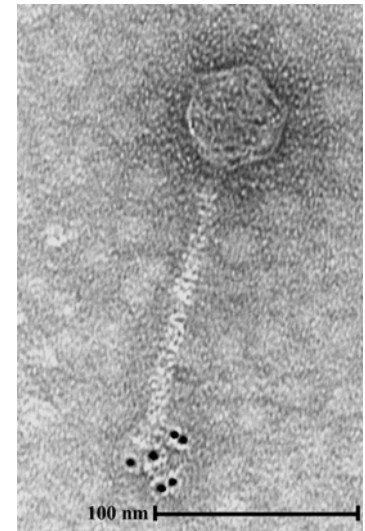
All isolated phages of *L. lactis* have double stranded DNA genomes and they belong to the order of tailed phages called *Caudovirales* <sup>79</sup>, which is divided into the families *Myoviridae* (long contractile tails), *Siphoviridae* (long non-contractile tails), and *Podoviridae* (short non-contractile tails). Each family is further divided into morphotypes depending on the head shape (isometric or prolate) and individual phages species are classified using DNA homology <sup>114</sup>. About half of all characterized phages and the majority of lactococcal phages are isometric headed and belong to the group of *Siphoviridae* <sup>1,17</sup>. Though lactococcal phages have been divided into ten different species <sup>38,56</sup>, the vast majority of phages isolated from dairy plants belong to the three species 936, c2, and P335 <sup>107</sup>.



Phages of the 936 and c2 species are strictly virulent whereas the P335 group contains both virulent and temperate species. The three species share very limited interspecies DNA homology and the P335 phages furthermore shows large intraspecies variation. The 936 and c2 species on the other hand has high intraspecies homology at >90% of their genomes<sup>24</sup>.

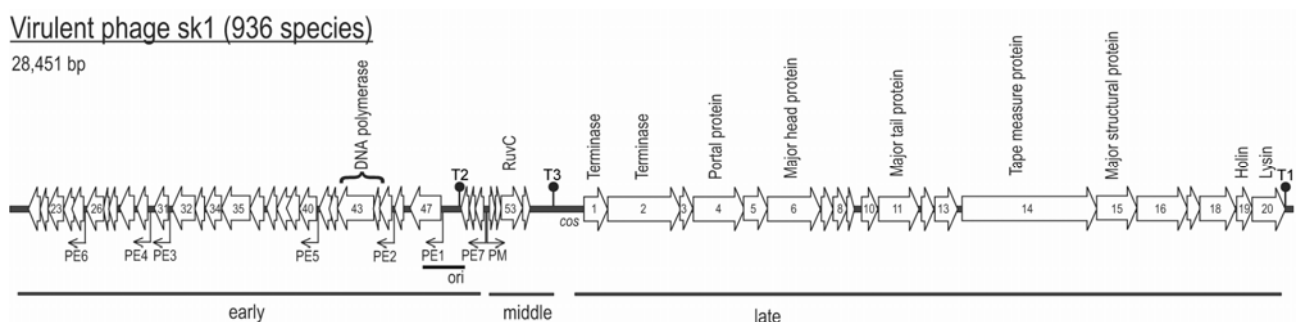
## PHAGE SK1 AS A REPRESENTATIVE OF 936-SPECIES

The results in this thesis are mainly based on studies on phages p2 and sk1 from the 936 species (Fig. 1). The genome of phage sk1 is characterized<sup>22,23</sup> and publicly available (GenBank acc.nr. NC\_001835). Temporal transcription data exists<sup>6,21</sup>, and the functions of several proteins have been determined. On the other hand, phage p2 was isolated more recently and less information is available, although comparative whole genome sequence analyses have revealed that sk1 and p2 are very close relatives (S. Moineau, unpublished data). In this thesis, data obtained using phage p2 is therefore compared to phage sk1 data which has a 28.5 kbp genome of the *cos* type like all other 936 phages<sup>17,23</sup>. The genome is divided into the three regions (early, middle, and late) according to the timing of transcription during the lytic cycle<sup>21</sup> (Fig. 2).



**Fig. 1.** Electron micrograph of phage sk1; adapted from Dupont *et al* (2004)<sup>48</sup>

The early region encompasses 30 small *orfs* of which several have overlapping stop/start codons. Sequence data of the overlapping genes suggested that several genes were coupled by programmed translational frameshift<sup>22,132</sup>. The functions of the early *orfs*, which are not encoding structural phage proteins, are unknown and a putative function based on DNA similarity has only been proposed for ORFs 43 and 44 (DNA polymerase, large and small subunit) and ORF35 (single strand DNA annealing protein). The early transcripts are observed from 2-5 minutes after infection



**Fig. 2.** The genomic map of phage sk1 showing *orfs* in the early, middle, and late regions. Arrows symbolize promoters. *Orf26* in the early region is the *sav* homologue of sk1; adapted from Brøndsted & Hammer (2005)<sup>17</sup>

and are repressed in the later phase of the lytic cycle <sup>21</sup>. Transcription of the early region probably occurs from several lactococcal consensus promoters, which are recognized by the host RNAP <sup>87</sup>. Three of the promoters were mapped by primer extension and promoter activity was further demonstrated for two of them by promoter fusion experiments <sup>22</sup>.

The middle region contains 4 ORFs, with a high degree of DNA similarity to the middle region of the 936 phage bIL66 <sup>8</sup>, where one of the ORFs previously was suggested to be a RuvC-like endonuclease <sup>9,17,30</sup>. The middle region is divergently oriented compared to the early region and transcripts appear 7-10 minutes after infection (Fig. 2). Transcription occurs from the promoter P<sub>M</sub>, which requires activation by an early expressed phage encoded protein. Deletion and mutagenesis analyses determined that the region from -36 to -55 upstream of the transcriptional start site was necessary for P<sub>M</sub> promoter activity <sup>22</sup>. The middle region is also expected to contain an activator of late gene transcription since late transcripts are not observed before 15 minutes after infection <sup>21</sup>. The authors also identified a terminator structure in the relatively large (ca. 1 kbp) non-coding region containing the *cos* site (Fig. 2). However, this terminator structure is not succeeded by the oligo(T) sequence (T-stretch) which is crucial for intrinsic transcription termination <sup>63</sup>. It is therefore likely that this terminator structure is inefficient and that this accounts for an observed read-through from the P<sub>M</sub> promoter <sup>21</sup>.

Transcription of the late region begins ca 15 minutes after infection, presumably after phage-mediated induction, though neither the late promoter nor the activator protein have been identified. The region encodes structural proteins as well as proteins needed for virion assembly and cell lysis (Fig. 2). The protein functions have been deduced from sequence similarity to other phage proteins for most of the late ORFs <sup>19,22,48</sup>. Only ORFs 19 and 20 were determined experimentally to encode the holin and lysin enzymes, respectively <sup>22</sup>. The gene order of the structural genes in the late region was highly similar to the order of the structural genes in phage  $\lambda$ , except for a few inversions <sup>22</sup>. However, phages are known to cluster genes of related functions together and the conservation might therefore reflect a functional more than an evolutionary relationship between the phages <sup>87</sup>.

## PHAGE BIOLOGY

### Initial infection

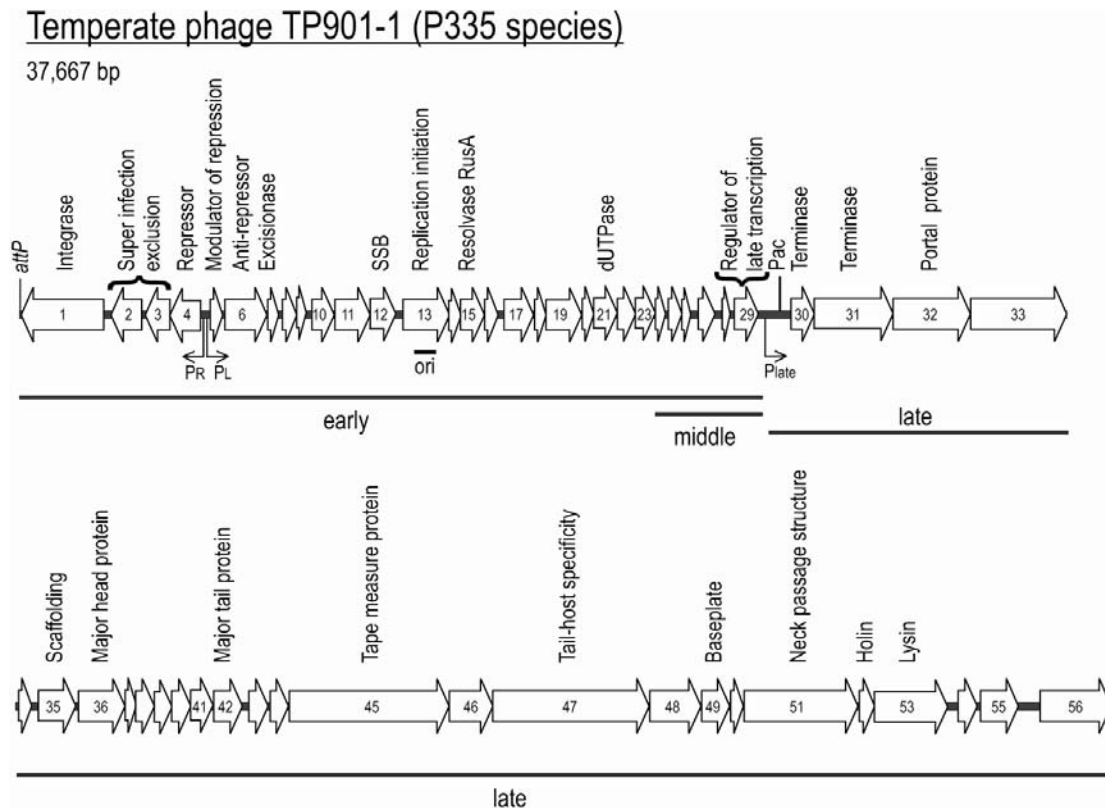
A phage infection starts with the adsorption of the phage to a cell. For several phages (presumably also lactococcal phages), the adsorption process happens in two steps: First, phage anti-receptors bind reversibly to carbohydrate structures allowing the phage to “walk” on the cell surface to obtain the correct position for binding irreversibly to a secondary receptor <sup>67,69</sup>. Following binding, the phage DNA is transferred to the cell cytoplasm by energy driven processes that require membrane potential <sup>87</sup>. The DNA transfer is not very well understood but it involves enzymatic degradation of PG and penetration of the cell membrane. The process differs among phage species and it is adapted to the general strategy of the infecting phage. Thus coliphage T7 transfers its DNA in a two-step process that involves “dragging” of the DNA into the cell using the host RNAP. The transfer is slow allowing expression of early proteins to protect sensitive DNA regions that are transferred later <sup>110,111</sup>. Other phages transfer DNA very quickly as for example in T4 where DNA enters the cell with a speed of 3-10 kbp per second <sup>113</sup>. Often a few proteins carried in the phage capsid are also transferred to the cell to mediate a fast take-over of the cellular machinery <sup>87</sup>. Mechanisms of DNA transfer in lactococcal phages are unknown.

Once the DNA has entered the cell, further development depends on the type of phage. All phages can be divided into two functional classes depending on their ability to integrate the phage genome into the bacterial chromosome. Phages with this ability are classified as temperate while phages lacking this ability are classified as virulent <sup>64</sup>.

### The lysogenic cycle

When the DNA of a temperate phage enters the bacterial cell it has the choice of entering the lytic or the lysogenic cycle, where the most frequent choice is to enter the lytic cycle. In the cases where the phage enters the lysogenic cycle a repressor protein (CI in phage  $\lambda$ ) prevents transcription from the promoter from which the lytic genes are transcribed. Instead the lysogenic module is expressed and the phage integrates into the host chromosome. For the lactococcal phage TP901-1 the lytic/lysogenic choice is determined by regulation of a genetic switch containing the two oppositely oriented promoters  $P_L$  and  $P_R$  <sup>17</sup> (Fig 3). Genes for the lytic cycle are transcribed from  $P_L$  <sup>96</sup>, whereas genes needed for establishing and maintaining lysogeny are transcribed from  $P_R$ . The switch is regulated by the protein CI (transcribed from  $P_R$ ) which represses both promoters and the

protein MOR (transcribed from  $P_L$ ) which counteracts the effect of CI. The relative amount of the two proteins determines the choice between lytic and lysogenic cycle<sup>97,98</sup>. If the phage enters the lysogenic cycle, an integrase mediates site specific integration at the *attB* site of the host chromosome<sup>16</sup>. The phage then exists as a passive prophage until an induction event, typically induced by the SOS response, and in which the host encoded RecA protein is needed, causes the phage to excise from the host chromosome and enter the lytic cycle<sup>15,97</sup>.



**Fig. 3.** The genomic map of the temperate phage TP901-1 showing *orfs* in the early, middle, and late regions. Arrows symbolize promoters. The region left of the  $P_R$  promoter is the lysogeny module; adapted from Brøndsted & Hammer (2005)<sup>17</sup>

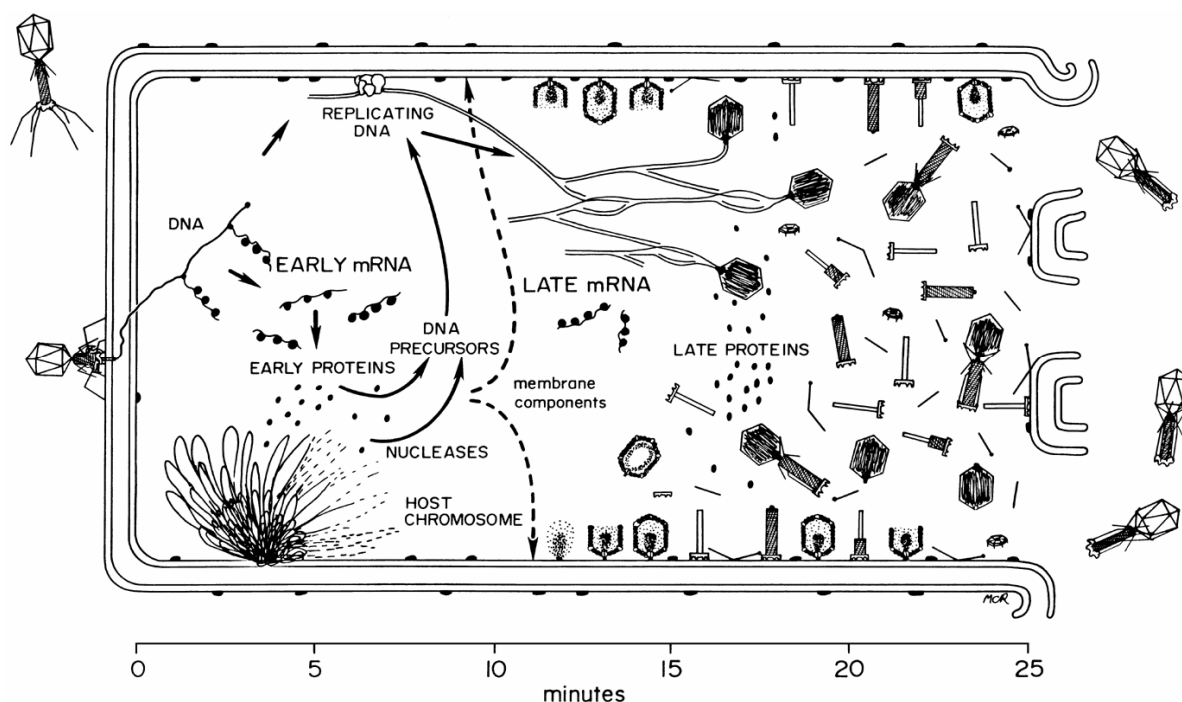
## The lytic cycle

The lytic cycle is a tightly regulated and efficient molecular machine that within very short time can produce and release complete infective phage particles from a single phage DNA molecule. The main processes are: replication of phage DNA, production of the structural proteins that will make up the virion, assembly of these proteins, packaging of phage DNA in the head, and finally the lysis of the host cell that releases the progeny phages to the environment. This process can in many phages be accomplished within 30 minutes<sup>87</sup>.

## The lytic cycle of coliphage T4

Insights into the molecular biology of the well characterized lytic cycle of coliphage T4 has revealed a complex and tightly regulated web of interactions that allows the phage to efficiently complete its lytic cycle while adapting to different situations imposed by the host or the environment<sup>82,113</sup> (Fig. 4).

As a general rule, phage T4, like many phages has clustered related genes together in early, middle, and late transcribed genes. The early genes are mainly used to shut-down cellular functions and redirect the cell to produce phage particles, the middle region controls timing of DNA replication as well as transcription of the late region, which is primarily encoding structural proteins<sup>87</sup>. Regulation is mainly at the transcriptional level, using modification of the host RNAP and sigma-factors to direct the timing of the transcription<sup>113</sup>. Thus, one of the first synthesized proteins in the T4 lytic cycle modifies the host RNAP to exclusively transcribe T4 DNA (which is distinguished from host DNA by the methylation of cytosine) thereby efficiently shutting off transcription of host genes<sup>46</sup>. For both early and middle transcription, T4 uses the host  $\sigma_{70}$  factor, which however gets modified by an early phage protein<sup>75</sup> to recognize middle instead of early promoters. Late



**Fig. 4.** The lytic cycle of coliphage T4. Different steps of the cycle are presented according to the timing of the lytic cycle (below picture). The complex regulatory mechanisms are not presented. Heads, tails, and tailfibers are prefabricated before assembly; adapted from Mosig & Eiserling (2005)<sup>113</sup>

transcription uses the phage encoded  $\sigma_{55}$  transcribed from the middle region<sup>83</sup> but many other factors are involved in the transition from middle to late transcription<sup>85</sup>. Late transcription is further mechanically coupled to DNA replication and packaging<sup>10</sup>, presumably as a way to match the amount of replicated DNA to the available phage heads.

The virion is assembled from prefabricated tails and heads filled with DNA, both of which have been synthesized at a membrane associated complex (Fig 4). Contrary to the early part of the lytic cycle where gene transcription is the regulating factor of phage development, the control of virion assembly lies in the ordered sequential assembly of proteins which are synthesized almost simultaneously<sup>91</sup>. Throughout the lytic cycle lysin and holin are produced and holin molecules gather on the inner cell membrane. Upon a signal, the holin produces a hole in the membrane that allows lysin to specifically adhere to and break down the PG layer after which the cell lyses quickly due to osmotic pressure<sup>53</sup>.

The size of the phage T4 genome is 170 kbp and it consists of about 300 genes of which about 160 have a known function. Some of the genes have been shown to be non-essential for phage proliferation under normal laboratory conditions but to be important for the adaptation of the phage to changing environmental conditions. This apparent “gene redundancy” has therefore been suggested to be an important parameter in the success of T4 as a molecular parasite<sup>113</sup>.

### **The lytic cycle of lactococcal phages**

The structure and assembly of the tail of phage TP901-1 and the closely related phage Tuc2009 are studied in details and they represent some of the best studied phage tails<sup>80,102,123,133,151,152</sup>. For most other lactococcal phages, the functions of the majority of the structural genes have been determined using sequence similarity and the molecular mechanisms of their lytic cycle are virtually unknown. Almost none of the early proteins of lactococcal phages show sequence similarity to proteins with known functions and therefore knowledge on gene regulation in the early lytic cycle is completely absent. The genomes of lactococcal phages are much smaller than the T4 genome (typically ca. 15-40 kbp). It can therefore be expected that they have less redundant genes since a larger fraction of the genome must be occupied by structural and other essential genes. On the other hand, redundancy has been demonstrated in the lysogenic module and near the *attP* site in temperate lactococcal phages. In the early region of the virulent phage sk1 some of the 30 non-structural genes are also redundant and many of these genes are expected to be involved in regulation of the lytic

cycle. The regulation of the lytic cycle in lactococcal phages might turn out to be a complex interaction network as observed in phage T4.

## **NATURAL PHAGE RESISTANCE MECHANISMS**

Lactococci contain a variety of natural anti-phage barriers to protect the bacterial populations against phage attacks. They are usually encoded on conjugal plasmids like most other factors required for optimized growth in milk. Due to the economical interest in *L. lactis*, research in phage resistance has mainly been focused on this species from which a number of phage resistance mechanisms have been isolated. Phage resistance mechanisms can be categorized into four different types according to where in the lytic cycle phage development is arrested. The four different types are: 1) inhibition of phage adsorption, 2) inhibition of phage DNA transfer to the cell, 3) restriction/modification (R/M) systems, and 4) abortive infection (Abi) mechanisms. The different phage resistance mechanisms have been reviewed extensively<sup>2,26,29,34,54,70,81,107,135</sup>.

### **Adsorption inhibition**

Inhibition of adsorption is thought to be the most frequent type of mutation in the isolation of bacteriophage insensitive mutants (BIMs) due to spontaneous mutations leading to changes in the phage receptor molecule on the bacterial cell wall<sup>70</sup>. Host-encoded adsorption inhibition mechanisms have also been isolated and this usually involves the production of cell surface antigens, or alteration or physical masking of the cell wall structures used for phage adsorption<sup>2,54</sup>. However, the general lack of knowledge of phage receptors on the lactococcal cell wall and the molecular mechanisms of the phage adsorption complicates the research in the adsorption inhibition phage resistance mechanisms<sup>2</sup>.

### **DNA transfer inhibition**

As for phage adsorption, the DNA transfer mechanisms into the cell are very poorly understood for lactococcal phages and thus the phage injection blocking resistance mechanisms also remain scarcely characterized<sup>54</sup>. The gene *pip* (phage infection protein) was shown to be important for irreversible attachment of c2-like phages as well as for DNA injection<sup>112</sup>. Pip is a membrane spanning protein but the cellular function is unknown since *pip*<sup>-</sup> strains show no reduction in growth potential<sup>58</sup>. Garvey *et al.* demonstrated a plasmid encoded DNA injection phage resistance

mechanism on the plasmid pNP40 (which also contains the abortive infection mechanisms AbiE and AbiF) <sup>59,60</sup>. Another injection blocking mechanism was demonstrated in the lysogeny module of the phage Tuc2009 and other temperate phages <sup>103</sup>. The gene *sie<sub>2009</sub>* encodes a protein which is associated with the cell membrane and cells expressing this gene were observed to adsorb 936 phages that were unable to transfer DNA into the cell.

### Restriction/Modification (R/M) systems

Once the phage DNA has entered the cell, restriction/modification (R/M) systems exist, that protect the cell against invading DNA. R/M systems constitute the vast majority of isolated bacterial phage resistance systems <sup>129</sup>, probably due the practical application of restriction enzymes as a tool in molecular biology. The R/M systems are widespread in lactic acid bacteria where they are usually located on plasmids <sup>81</sup>. They function by degrading foreign incoming DNA, which is distinguished from cellular DNA by methylation of cytosine or adenine at the recognition site on the cellular DNA. R/M systems are divided into general types depending on subunit composition and mode of action (Table 1). R/M systems are not 100% efficient which is a problem since DNA of a phage that escapes a R/M system will be methylated thereby offering complete protection against the given system <sup>54,81</sup>. Other phage strategies to circumvent R/M systems include: elimination of restriction sites on the phage genome, acquisition of methylase genes, and expression of endonuclease inhibitors <sup>54</sup>. Therefore, single R/M systems alone are not very powerful and are often used in combination with other R/M systems or abortive infection (Abi) systems <sup>81</sup>.

**TABLE 1.** Different types of R/M systems

	Type I	Type II	Type III
<b>Structural genes</b>	<i>hsdR</i> , <i>hsdM</i> , and <i>hsdS</i>	<i>R</i> and <i>M</i>	<i>mod</i> and <i>res</i>
<b>Characteristics</b>	All three proteins form a multifunctional multisubunit complex. HsdS and HsdM are responsible for recognition and methylation, respectively.	R is a restriction enzyme while M is a modification enzyme. R and M do not function as a multisubunit	Mod recognizes and modifies DNA while Res in complex with Mod is responsible for cleavage
<b>DNA cleavage</b>	Cleavage occurs far from recognition site	Cleavage within a symmetrical recognition site	Cleavage occurs at a fixed distance from recognition site



### **Abortive infection (Abi) systems**

Whereas the R/M systems protect the bacterium against any invading foreign DNA, abortive infection mechanisms only protect the bacterial population against attacks from specific phages. Abi systems constitute the last barrier for the bacterial population against phage proliferation and they always cause the death of the individual cell. However, since the cell does not release progeny phages (or only very few), the invading phage particles are titrated and the bacterial population survives. This general feature of Abi mechanisms has led to the suggestion that Abi systems are altruistic phage resistance mechanisms. However, the general lack of knowledge on the mode of action in Abi systems prevents scientists to determine if Abi systems are designed to kill the cell or if cell death is just a secondary effect in a process designed to arrest phage development<sup>2,134</sup>. Abi systems exist in many bacterial species<sup>5,27,90,136,137,148,68</sup> but the majority of the described Abi systems have been isolated in *L. lactis*<sup>2</sup>. They share several phenotypic characteristics: Efficiency of plaquing (EOP) is reduced to values ranging from  $10^{-1}$  to  $<10^{-9}$ , the efficiency of infection form (ECOI) is reduced, burst size is reduced, and latency time might be prolonged, leading to a reduction in plaque size<sup>70</sup>. Furthermore, Abis are characterized by an unusually low G+C content (24-31 %) compared to the average 37 % of lactococcal genes<sup>54</sup>. This has been suggested to be caused by horizontal gene transfer from other bacterial species<sup>117</sup>, or by codon bias in the Abi genes resulting in the use of rare tRNA species<sup>26</sup>. The majority of lactococcal Abi systems are encoded by a single gene but a few consist of two<sup>11,32,36,59,117</sup> or three genes<sup>150</sup>. Abi proteins generally lack secretion signal sequences and membrane spanning domains, and they contain hydrophilic charged residues, which indicate that they are present in the cytosol of the cell<sup>2</sup>. Only a few Abi systems are found on the chromosome of the host<sup>28,65,124,126</sup>. Thus the majority of the Abi mechanisms are located on plasmids, which are often conjugative<sup>135</sup>, thereby facilitating the sharing of anti-phage barriers among bacterial populations<sup>26</sup>.

The regulation of Abi systems is not very well understood<sup>2</sup>. The genes encoding AbiA and AbiB are expressed from constitutive promoters<sup>28,73</sup>, while regulation of a toxic Abi protein has been demonstrated for AbiD1, AbiK, AbiN, and AbiO<sup>26,3,52</sup>. It can be speculated that the latter systems are designed to kill the bacterium upon expression induced by the phage infection whereas the constitutively expressed proteins either function by inhibiting phage development directly or they need activation by phage encoded factors to become toxic for the host cell. In all cases, the Abi systems interact with phage factors involved in the infection to ultimately halt phage proliferation.

Though Abi systems share common phenotypic characteristics, they likely share very few similarities in their molecular mode of action (MOA). For most Abi systems, the MOA has only been investigated at a general level and the few studies where more detailed information of the molecular MOA has been obtained, data indicates that the term Abi mechanism covers a wide range of molecular un-related interactions which have been grouped together by scientists in a “black box” called abortive infection<sup>26</sup>. The apparent diversity in molecular MOA is not surprising given that the lytic cycle of phages is a complex web of temporally regulated interactions between components of both host and phage, as described above<sup>113</sup>. Some Abi mechanisms, have even been shown to differ in their MOA depending on which phage is infecting<sup>3,14,26,65</sup>, which supports the apparent complex molecular nature of the interactions between Abi systems and infecting phages.

### **Mode of action of lactococcal Abi systems**

Traditionally, Abi system mode of actions have been characterized as early or late depending if the system halts phage proliferation before or after DNA replication<sup>54,59</sup>. According to this definition AbiA<sup>71</sup>, AbiF<sup>59</sup>, and AbiR<sup>150</sup> are early Abi mechanisms while AbiK<sup>14</sup> and AbiV<sup>Manuscript I+III</sup> are “partly early” mechanisms since they inhibit DNA replication of P335 and c2 phages, respectively while allowing DNA replication of 936 phages. Most other Abi systems have been classified as late<sup>11,50,54</sup>. It can be difficult to determine the exact MOA of Abi systems since the primary effect on the Abi target is followed by a range of deleterious physiological effects in the cell, which can be hard to separate from the primary effects<sup>26</sup>. Therefore, the use of Abi-insensitive phage mutants can be a valuable tool in elucidating the molecular MOA of individual Abi systems. With a few exceptions these phage mutants can be obtained and by using genomic analyses, the mutated phage gene(s) rendering the phage insensitive to Abi systems can subsequently be determined. Using this approach, genes involved in the Abi sensitivity of lactococcal P335-like phages have been identified either by specific point mutations in the genome of phage escape mutants or by exchange of genetic material with a prophage<sup>11-13,40,89,108</sup>. In the case of virulent 936 phages, Abi sensitivity genes were identified by point mutations in the escape phage mutants<sup>7,8,11,40,54</sup> or by recombination between two virulent phages<sup>45</sup>. Among these genes there is a prevalence of early expressed phage genes, which remain virtually uncharacterized, thereby complicating the process of revealing the MOA of a given Abi system.

## Individual Abi systems

Several Abi systems have been characterized to some detail regarding the molecular mode of action (see Table 2 for a summary of the different Abi characteristics). For example, AbiC was shown to contain putative transmembrane helices and to cause a ca 50% reduction in major capsid protein production<sup>49</sup>. AbiQ as demonstrated for many Abi systems allowed DNA replication of 936 and c2 phages, however only the concatemeric form of DNA was observed. In addition, synthesis of the major capsid protein was observed and the authors speculated that defective assembly possibly due to insufficient packaging of the concatemeric phage DNA might be involved in the MOA of AbiQ<sup>51</sup>. The effect of AbiR that works by impeding phage DNA replication is dependent on the methylase LlaKR2I which is part of a R/M system located on the same plasmid as AbiR. This methylase protects the host against a toxic effect of the Abi system<sup>150,156</sup> and AbiR functions whether methylated or non-methylated phage DNA is used for infection. This made the authors suggest that the MOA could involve inhibition of DNA replication initiation if the *ori* was not methylated<sup>156</sup>.

Some Abi systems have been shown to interfere with transcription of phage genes. For example a northern analysis of phage RNA in AbiU containing cells suggested that transcription was delayed for 936 phages and c2 phages<sup>32</sup>. Interestingly, transcription delay was also shown for 936 phages in cells harbouring AbiG<sup>117</sup> and it was noted that AbiG and AbiU both are two-component systems that even appear to be related on the basis of sequence similarity<sup>32</sup>. The last Abi system that has been shown to interact with transcription is AbiB which is one of the few known chromosomally encoded Abi systems<sup>28</sup>. This Abi system is only effective against 936 phages and it is expressed constitutively from a promoter in a nearby *ISS1* element though overexpression is toxic for *L. lactis* and *E. coli*<sup>26</sup>. DNA replication and RNA transcription was demonstrated to occur normally until 10-15 minutes after infection. At this time a dramatic decay of phage transcripts was observed in AbiB containing cells<sup>122</sup>. Though the MOA was not determined at the molecular level, the effect, which arrested phage proliferation, was suggested to be caused by RNase activity. By blocking phage protein synthesis at different time points during infection, the authors further demonstrated that the decay was induced by an early phage protein which was suggested to either activate or induce the production of the RNase<sup>122</sup>.

**TABLE 2.** Characteristics of lactococcal Abi systems

Name and source	Effective against <sup>a</sup>			Characteristics of gene and gene product	Effect on phage lytic cycle <sup>b</sup>	Year	Ref <sup>c</sup>
	936	c2	P335				
<b>AbiA</b> pTR2030, <i>L. lactis</i> ssp. <i>lactis</i> ME2	sk1	c2	ø31	Constitutively expressed. Activity is heat-sensitive and gene-dosage dependent Leucine-rich repeat essential for Abi phenotype. Protein similarity (23%) to AbiK	Affects DNA replication and phage capsid production. Two regions on phage $\Phi$ 31, including a homologue to a DNAP associated protein, involved in AbiA sensitivity. Functions also in <i>Streptococcus</i>	1989	39- 41,73,142
<b>AbiB</b> pHP003, <i>L. lactis</i> ssp. <i>lactis</i> IL416	bIL170 bIL66	no	no	Constitutive expression from iso-ISSJ element.	Phage transcripts degraded 10-15 min after infection. AbiB has direct or indirect RNase activity. DNA replication not affected.	1991	28,122
<b>AbiC</b> pTN20, <i>L. lactis</i> ssp. <i>lactis</i> ME2	p2 jj50 sk1	no	ul36 m12r	Two membrane spanning domains. Activity is gene-dosage dependent. Protein similarity (22%) to AbiP	Normal DNA replication, 50% reduction in production of major capsid protein (MCP)	1992	49
<b>AbiD</b> pBF61, <i>L. lactis</i> ssp. <i>lactis</i> KR5	sk1	c2	no	Expression may be regulated. Activity is heat-sensitive. Protein similarity (28-41%) to AbiD1 and AbiF	Normal DNA replication of sk1	1995	106
<b>AbiD1</b> pIL105, <i>L. lactis</i> ssp. <i>cremoris</i> IL964	bIL170 bIL66	c6A bIL67	no	Strong constitutive expression but quick termination unless induced by phage. Toxic when overexpressed. Two putative DNA-binding HTH domains. Protein similarity (28-41%) to AbiD1 and AbiF	Induced by middle expressed phage protein Orf1 (only present in 936 phages). Causes inhibition of translation of phage Ruv-C like endonuclease. Blocks resolution of DNA structures and packaging	1995	3,7,8
<b>AbiE</b> pNP40, <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> DRC3	712	no	no	Two overlapping genes ( <i>abiEi</i> and <i>abiEii</i> )	Normal DNA replication	1995	59
<b>AbiF</b> pNP40, <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> DRC3	712	c2	no	Protein similarity (28-41%) to AbiD1 and AbiF	Decreased rate of DNA replication. Chromosomally encoded RecA needed for AbiF phenotype	1995	59,61

**TABLE 2.** Characteristics of lactococcal Abi systems

Name and source	Effective against <sup>a</sup>		Characteristics of gene and gene product		Effect on phage lytic cycle <sup>b</sup>	Year	Ref <sup>c</sup>
	936	c2	P335				
<b>AbiG</b> pCI750, <i>L. lactis</i> ssp. <i>cremoris</i> UC653	712	(c2)	no	Two genes ( <i>abiGi</i> and <i>abiGii</i> ) in two-gene operon. Protein similarity (43%) between AbiGii and AbiU2	Normal DNA replication. Inhibition of RNA synthesis	1996	117,118
<b>AbiH</b> chromosome of <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> S94 and S96	Φ59	(Φ53)	no	Possible IS-like element downstream of gene	Unknown	1996	124
<b>AbiI</b> pND852, <i>L. lactis</i> ssp. <i>lactis</i> M138	712	c2	no	Unknown	Normal DNA replication but no packaging	1997	141
<b>AbiJ</b> pND859, <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> UK12922	(712)	no	no	Putative DNA-binding HTH motif	unknown	1997	35
<b>AbiK</b> pSRQ800, <i>L. lactis</i> ssp. <i>lactis</i> W1	p2 sk1 jj50	(c2) (ml3) (eb1)	ul36 Q30 Q33	Regulated expression, toxic to the host. Heat-sensitive and gene-dosage dependent activity. Protein similarity (23%) to AbiA	Normal DNA replication (concatemeric) of 936 phages, No DNA replication in P335 phages. Normal RNA synthesis in p2 but blocking of late gene expression in ul36. Target in both 936 and P335 phage species is a phage ssDNA recombinase. Reverse transcriptase domain of AbiK is essential for activity.	1997	13,14,52,55
<b>AbiL</b> pND861, <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> UK12922	712	(c2)	no	Two-gene operon ( <i>abiLi</i> and <i>abiLii</i> ) constitutively expressed. Protein similarity (28-41%) to AbiEi+ii and AbiGi+ii.	No effect on gene transcription in c2	1999	36
<b>AbiN</b> chromosome of <i>L. lactis</i> ssp. <i>cremoris</i> S114	Φ59	(Φ53)	no	Suggested toxicity when overexpressed, located in region containing prophage genes	Unknown	1998	126

**TABLE 2.** Characteristics of lactococcal Abi systems

Name and source	Effective against <sup>a</sup>			Characteristics of gene and geneproduct	Effect on phage lytic cycle <sup>b</sup>	Year	Ref <sup>c</sup>
	936	c2	P335				
<b>AbiO</b> pPF144, <i>L. lactis</i> ssp. <i>lactis</i> S45-91-1	Φ59	(Φ53)	no	No promoter or RBS found upstream of <i>abiO</i> . Suggested toxicity when overexpressed	Unknown	1998	125
<b>AbiP</b> pIL2614, <i>L. lactis</i> ssp. <i>lactis</i> IL420	bIL66M1 bIL41 bIL170 sk1	no	no	Protein similarity (22%) to AbiC. Gene-dosage dependent. Maybe regulated by co-expressed <i>orf1</i> . Putative membrane anchored protein	DNA replication arrested ca. 10 min after infection. Lower levels of middle and late transcripts. Early transcripts not switched-off. An early phage gene confers AbiP insensitivity	2004	44,45
<b>AbiQ</b> pSRQ900, <i>L. lactis</i> ssp. <i>lactis</i> W-37	p2 sk1 jj50 bIL170 P008	c21 ml3 eb1	no	Heat stable activity. Expression may be regulated	Normal DNA replication (concatemeric) and production of major capsid protein (MCP). Targets different genes in sensitive 936 phages	1998	51,131
<b>AbiR</b> pKR223, <i>L. lactis</i> ssp. <i>lactis</i> biovar. <i>diacetylactis</i> KR2	no	c2	no	Encoded by a three gene operon. Toxic without the methylase from adjacent R/M system. Heat sensitive activity. Flanked by IS982 elements.	Impedes DNA replication	2000	150,156
<b>AbiS</b> pAW601, <i>L. lactis</i> ssp. <i>cremoris</i> W60	(jj50) (sk1) (p2)	no	no	Unknown	Non-coding sequences with homology to <i>cos</i> site involved. Action of AbiS-like system or R/M system increases efficiency.	2004	76,81
<b>AbiT</b> pED1, <i>L. lactis</i> ssp. <i>lactis</i> W51	p2 p2k sk1 jj50	no	ul36 Q30 Φ31 Φ50	Two constitutively co-expressed genes ( <i>abiTi</i> and <i>abiTii</i> ). Gene-dosage dependent activity that requires putative transmembrane helix.	DNA replication (concatemeric) though lower level compared to wt. No effect on gene transcription. Production of capsids, but no DNA packaging. Probably interacts with late genes in ul36	2002	11
<b>AbiU</b> pND001, <i>L. lactis</i> ssp. <i>lactis</i> LL51-1	(712)	(c2)	(ul36)	Two genes ( <i>abiU1</i> and <i>abiU2</i> ). Protein similarity (43%) between AbiU2 and AbiGii. Expression probably regulated.	Gene transcription is delayed. AbiU1 is primary determinant for Abi phenotype while AbiU2 appears to downregulate the AbiU effect.	2001	32

**TABLE 2.** Characteristics of lactococcal Abi systems

Name and source	Effective against <sup>a</sup>		Characteristics of gene and gene product		Effect on phage lytic cycle <sup>b</sup>	Year	Ref <sup>c</sup>
	936	c2	P335				
<b>AbiV</b> chromosome of <i>L. lactis</i> ssp. <i>cremoris</i> MG1363	sk1 p2 jj50 P008 bIL170	c2 bIL67 ml3 eb1	no	Silent on chromosome. Non toxic when over-expressed. Gene-dosage dependent activity against c2-species. Heat-stable. Dimer in native form. Contains putative RNA binding site	DNA replication in 936 (concatemeric) but not in c2. Middle and late transcripts reduced. Complete inhibition of protein synthesis. Interacts directly with early transcribed phage protein dimer with antimicrobial activity.	2008	I, II, III
<b>AbiZ</b> pTR2030, <i>L. lactis</i> ssp. <i>lactis</i> NCK391	no	no	<p> <math>\Phi</math>31 ul36 Q30 Q33 Q36 phi48 phi50 </p>	<p>           Contains two putative transmembrane helices.            Located in putative mobile region flanked by IS-elements </p>	<p>           Causes premature (15 min) cell lysis through indirect interaction with the phage encoded holin protein. An early or middle transcribed phage gene is essential for the function of AbiZ. </p>	2006	50

<sup>a</sup> The specific phage subspecies against which the system has been tested to be efficient are listed. Paranthesis indicate that the Abi mechanism is categorized as weak ( $10^1$ - $10^3$ )<sup>107</sup> and thus only shows limited efficiency against the given phage.

<sup>b</sup> DNAP is DNA polymerase

<sup>c</sup> I, II, and III represents Manuscripts I, II, and III, respectively.

AbiA was the first Abi system to be isolated by Klaenhammers group<sup>73,74</sup>. It is active against the three main lactococcal phage species 936, c2, and P335 and it prevents DNA replication. It is encoded on the conjugative plasmid pTR2030 from which it is expressed constitutively<sup>73</sup>. AbiA efficiency against all three main phage species is increased with higher gene expression<sup>39</sup>. Interestingly, as the first Abi system to cross a bacterial species barrier, AbiA was demonstrated to be active against phages of *Streptococcus thermophilus*, which is a close relative to *L. lactis*. AbiA is temperature sensitive in both *S. thermophilus* and *L. lactis* with no phage resistance effect at 37°C<sup>142</sup>. The system causes almost complete inhibition of phage capsid production, probably as a secondary effect of the early inhibition of DNA replication. The central part of AbiA contains a leucine repeat which was demonstrated by site directed mutagenesis to be required for the phage resistance function of the protein. This leucine repeat was speculated to be involved in either homodimer formation or heterodimer formation with a phage encoded target protein<sup>41</sup>. Different AbiA targets were determined by genetic analyses of AbiA insensitive phage mutants. One mutation was found to cause a arginine to leucine change in the ORF245 on the Φ31 genome<sup>40</sup>. This gene shows sequence similarity to DNA polymerase accessory proteins and might therefore be involved in the DNA replication of Φ31. In an independently isolated phage Φ31 mutant, mutations were found in an intergenic region containing two inverted repeats upstream of ORF245 and complementation experiments coupled this region to the action of AbiA<sup>40</sup>. The authors suggested that the region might function as a false target for AbiA. Interestingly, the genetic regions providing the AbiA targets found in Φ31 are not present in phage c2 and it was therefore suggested that AbiA might work with different MOAs against P335 and c2 phages<sup>40</sup>.

AbiA shares 23 % protein sequence similarity with AbiK which is also efficient against all the three main lactococcal phages and works by preventing DNA replication in at least P335 phages<sup>14,52</sup>. AbiK is also sensitive to higher temperatures and the efficiency of the system was demonstrated to depend on copy number. No phage proteins were produced in AbiK containing cells which was probably a secondary effect of the inhibited DNA replication. The many similarities to AbiA led the authors to suggest that AbiA and AbiK belong to the same protein family<sup>52</sup>. Furthermore, it was shown that AbiK insensitive phage mutants carry mutations in proteins that are related to single strand annealing proteins<sup>13</sup> and thus are functionally related to the AbiA target<sup>2</sup>. Interestingly, phage mutants of the 936 species are also mutated in genes encoding single strand annealing proteins even though the MOA of AbiK was suggested to be different in this phage species due to



observed DNA replication<sup>13,26</sup>. More recently, AbiK was demonstrated to be part of an operon containing a phage related transcriptional repressor suggesting regulation of the *abiK* transcription<sup>55</sup>. A reverse transcriptase region was also discovered in AbiK which was demonstrated by site-directed mutagenesis to be essential for the Abi phenotype. A model was proposed to explain the MOA of AbiK. This included the synthesis of a single-stranded cDNA molecule that could titrate the mRNA from which phage single strand binding proteins are encoded, thereby inhibiting DNA replication. This however, cannot explain the AbiK phenotype of an infection with 936 phages in which DNA replication and early transcription is not affected by AbiK. The authors suggested protein synthesis inhibition to be the MOA of AbiK against these phages<sup>55</sup>.

Another Abi system interfering with DNA replication is AbiP. This protein is encoded in a two-gene operon and is only effective against 936 phages<sup>45</sup>. AbiP is a membrane-anchored protein which is able to bind ssRNA or DNA in a sequence dependent manner. It shares 22 % amino acid sequence similarity with AbiC<sup>45</sup>. In cells harbouring AbiP, DNA replication is halted 10 minutes after infection which probably causes the observed lower levels of middle and late transcription. During a normal infection with 936 phages, early transcription is switched off ca 15 minutes after infection. This was not observed in AbiP containing cells, where the level of early transcription continued to rise throughout the experiment. An early transcribed phage gene involved in sensitivity to AbiP was determined and in the same study it was demonstrated that virulent phages may acquire resistance to Abi mechanisms by homologous recombination during co-infection with a phage which has a high degree of nucleotide sequence similarity<sup>44</sup>.

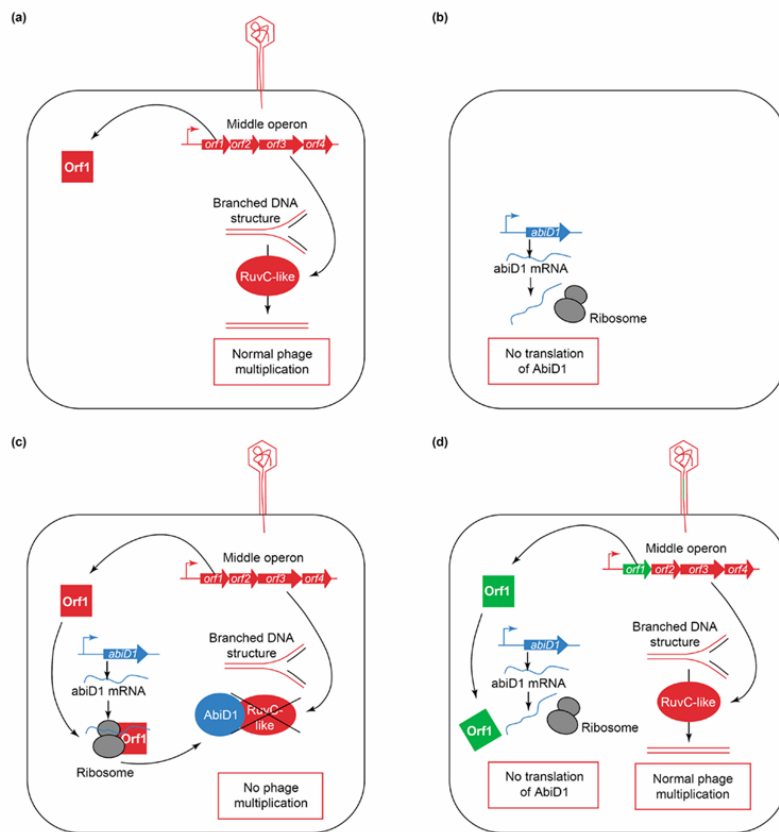
AbiT also consists of a two gene operon which is constitutively expressed and it affects phages from the 936 and P335 species<sup>11</sup>. As for several other Abi systems, increased expression causes increased efficiency (EOP) against the tested phage species. The level of DNA replication was lowered in cells harbouring AbiT and only concatemeric DNA existed, indicating that the MOA is arresting phage development after DNA replication and that secondary effects cause the lower level of DNA replication. Gene expression was measured using Northern Blotting for an early and a late gene observing no difference in cells with or without AbiT. It was therefore suggested that AbiT affects the phage cycle at a late stage. Electron microscopy revealed the assembly of phage capsids but the phage morphogenesis was inhibited before correct assembly and lysis. Site directed mutagenesis was used to demonstrate that a putative membrane spanning domain of AbiT was

essential for the Abi phenotype. An AbiT insensitive phage mutant was analyzed and it was found that a late phage protein is essential for the Abi phenotype probably by activating AbiT which then causes rapid and premature cell death. It was speculated that the membrane spanning domain is involved in making pores in the membrane to cause the premature lysis as observed in the Rex Abi systems of *E. coli*<sup>137</sup>.

Another Abi system also functioning by premature killing of the host cell is AbiZ<sup>50</sup>. This Abi protein which was recently discovered on the pTR2030 plasmid that also harbours AbiA and a R/M system is effective against P335 phages but not phages from the 936 or c2 species. Two putative transmembrane helices were found in the protein which as for AbiT were suggested to be involved in the phage resistance phenotype. AbiZ causes premature lysis (15 minutes early) of the infected cells. Nisin induced expression of holin and lysin caused partial lysis of cells without AbiZ whereas the presence of AbiZ caused lysis to occur 30 minutes earlier while also increasing the membrane permeability. This made the authors suggest that AbiZ may interact with holin to cause the premature lysis<sup>50</sup>. However, through sequence analysis of AbiZ insensitive phage mutants, it was discovered that an early or middle phage encoded factor is involved in the AbiZ phenotype since mutant phages had exchanged the complete middle and late region of the genome and no mutations were found in either holin or lysin<sup>50</sup>.

Probably the best understood lactococcal Abi system with regards to molecular MOA is AbiD1<sup>3,7,8,26</sup>. This is a plasmid encoded Abi system that is effective against phages from the 936 and c2 species. High expression of the gene is toxic to *L. lactis* and it is therefore tightly regulated at the transcriptional<sup>3</sup> and translational<sup>26</sup> level. The protein contains two putative HTH motifs and is highly charged with a pI of 10.4 which indicates that the protein is involved in DNA binding in the cytosol. The phage middle region of the genomes of 936 phages was demonstrated to be essential for AbiD1 function since complementation with a mutated middle region abolished the Abi phenotype. The middle region consists of 4 genes which are transcribed from the phage activated middle promoter. Independently obtained point mutations in the *orf1* gene of this region strongly indicated this gene product being essential for the AbiD1 function<sup>8</sup>. The *abiD1* gene is transcribed from a strong promoter and the tight transcriptional regulation is imposed by a very efficient transcription termination ca 50 bp downstream of the promoter<sup>3</sup>. It is therefore possible that ORF1 functions as an anti-termination protein that allows fast and high expression of AbiD1 upon phage

infection. In support of this, AbiD1 efficiency was shown to be positively correlated to the expression level of ORF1 but did not depend on expression of a mutated ORF1<sup>7</sup>. The ORF1 was suggested to exhibit a dual function where the N-terminal part is important for interaction with AbiD1 whereas the C-terminal part interacts with the downstream ORF3. This gene has a high degree of amino acid sequence similarity to the *E. coli* RuvC endonuclease and it is likely that the protein is involved in resolving branched DNA generated during DNA replication. The ORF3 endonuclease, which is essential for phage proliferation, was also demonstrated to alleviate the effect of AbiD1 when expressed in *trans* suggesting that AbiD1 functions by decreasing the amount of ORF3<sup>8</sup>, thereby interfering with phage DNA packaging. Interestingly, expression in *trans* of ORF3 from phage bIL66 (936-phage) allows bIL67 (c2-phage) to proliferate in cells containing AbiD1 thereby demonstrating ORF3 being the target for AbiD1 in these two different phages. However, the induction process must differ since bIL67 does not contain an *orf1* homologue<sup>26</sup>. The authors constructed a model to explain the molecular MOA of AbiD1 (Fig. 5).



**Fig. 5.** Model for AbiD1 mode of action. (a) Following infection of *AbiD1*<sup>-</sup> cells, phage bIL66 produces an essential RuvC-like endonuclease, which resolves branched DNA structures generated during phage DNA replication. (b) In *AbiD1*<sup>+</sup> cells, *abiD1* translation is repressed under normal conditions. (c) Following infection of *AbiD1*<sup>+</sup> cells, Orf1 induces *abiD1* translation, resulting in inhibition of the RuvC-like endonuclease activity and arrest of phage multiplication. (d) bIL66 *AbiD1*-resistant mutants all produce a mutated Orf1, which is unable to induce *abiD1* translation. Phage multiplication proceeds normally; adapted from Chopin *et al.* (2005)<sup>26</sup>

## ***E. COLI* PHAGE EXCLUSION MECHANISMS**

A few Abi systems (also referred to as phage exclusion proteins) have been well described in *E. coli* and reviewed by Snyder<sup>137</sup>.

### **The Rex system**

The Rex exclusion system is encoded by phage  $\lambda$  presumably to protect the prophage against invading virulent phages as observed for the lactococcal Sie systems<sup>103</sup>. The system consists of the two gene products RexA and RexB which are cytoplasmic and transmembrane proteins, respectively. It was shown that the lytic cycle of phage T4 *rII* mutants would proceed normally until the onset of DNA replication at which time the membrane potential of the cell drops and the cell dies causing the arrest of phage development. Although the specific molecular mechanism is unknown, a protein-DNA complex, possibly a replication or recombination complex was suggested to trigger the sudden decrease of membrane potential, which was mediated by RexB forming an ion channel allowing passage of monovalent cations<sup>137</sup>. The similarities between the RexA/RexB system and the lactococcal Abi system AbiT are interesting. Thus both systems are two component systems containing a membrane spanning protein and a cytosolic protein, and both systems work relatively late by sudden cell death<sup>11,137</sup>.

### **The Lit and PrrC systems**

Two other phage exclusion systems have been described in *E. coli* both of which have comparable patterns of MOA. Both systems consist of a constitutively expressed inactive protein which is activated upon phage infection by a small phage encoded peptide. Once activated, the protein interferes with evolutionary conserved and essential components of the cell translation machinery by cleaving in one case an elongation factor and in the other case the tRNA<sup>Lys</sup><sup>137</sup>. In the former case, the Lit protein is activated by a short peptide determinant located in the major head protein (MHP) which is expressed late in the lytic cycle of phage T4. The presence of Lit simultaneously induces transcription termination of the MHP thereby arresting phage assembly and hindering folding of the native MHP protein which will leave the small inducing peptide region exposed for activation of Lit proteins. The resulting Lit cleavage of the EF-Tu elongation factor arrests translation and thus phage development.

The second system aborts infection of polynucleotide kinase or RNA ligase deficient mutants of T4 by the activity of the *prrC* gene which encodes a ribonuclease that specifically cleaves the host lysine tRNA<sup>137</sup>. The PrrC is activated by the small (26 amino acids) phage encoded peptide Stp which is redundant for normal proliferation of T4. The apparent paradox as to why the phage would encode a non-essential protein which only known function is to inhibit phage proliferation was solved and gives an insight into the complex molecular interactions in the ongoing arms race between phage and host. The *prrC* gene is located inside a Type Ic R/M restriction cassette between the *hsdS* and *hsdR* genes and the PrrC protein interacts physically with the HsdS and HsdR proteins. In this configuration and in the absence of phage infection the activity of PrrC is not observed, but cloning and expression of a solitary *prrC* revealed an active enzyme. It was therefore suggested that the physical interaction between the R/M proteins and PrrC masks the deleterious effects of the endonuclease. The real target of the phage encoded Stp might therefore not be the PrrC but the R/M system. Since Type I R/M systems need to form a complex to be active, the Stp might function by dissociating the R/M complex thereby releasing the PrrC protein that subsequently halts translation and phage proliferation by cleaving the lysine tRNA. Thus, the PrrC Abi system possibly functions as a second line of defence to arrest phage development if the R/M system is bypassed by an infecting phage<sup>137</sup>.

It is interesting to note that the MOA of AbiD1 resembles the general pattern of the Lit and PrrC systems. The majority of investigations carried out on the MOA of lactococcal Abi systems have only been performed for one of the three main phage species. This is unfortunate since the MOA of several lactococcal Abi systems differs according to which phage is infecting<sup>8,14,26,40, Manuscript III</sup>. In general, more research focused on the MOA of lactococcal Abi systems is needed to reveal the underlying molecular mechanisms of these interesting phage resistance barriers.

## **OTHER PHAGE RESISTANCE MECHANISMS**

### **Engineered phage resistance**

Although natural phage resistance mechanisms constitute the majority of known anti-phage barriers, the persistence of industrial phage problems combined with increased availability of phage and bacterial genome data and development of molecular tools, has made it feasible to attempt to engineer phage resistance mechanisms for protection of industrial production strains<sup>29,81,107</sup>. One

approach is to produce antisense mRNA by transcription of essential phage genes on the non-coding DNA strand. In theory, the antisense mRNA will hybridize to the mRNA thereby blocking transcription and therefore also phage development <sup>104</sup>. However, in general this method has been demonstrated to have only very limited effect on phage inhibition <sup>84</sup>.

The system designated PER for phage encoded resistance utilizes the cloning of a bacteriophage origin of replication (*ori*) on a plasmid. Upon phage infection, the alternative *ori* will function by competitive inhibition, titrating the phage replication factors and thereby inhibit phage proliferation <sup>72</sup>. As expected the effect was shown to be dependent on the copy number of the plasmid. The system has been applied as a defence against different lactococcal phages <sup>104,105,119</sup> but generally, PER systems suffer from acting very specifically against phages with a specific *ori*.

Another engineered phage resistance system takes advantage of phage promoters which are activated at a late stage of the lytic cycle by a phage encoded activator molecule. These promoters are not recognized by the host RNAP and if a toxic gene is cloned after such promoters it will work as a phage induced suicide mechanism. This was exploited and patented by Klaenhammers group <sup>43</sup>. The system was improved with respect to promoter strength and copy number as well as an enhancer of restriction activity <sup>42</sup> and also combined with a PER system specific for  $\Phi 31$  in addition to AbiA. By stacking the phage resistance mechanisms the occurrence of insensitive phage mutants was completely eliminated.

A problem with these engineered phage resistance mechanisms is that generally they are less efficient against the target phages and they only function against a narrow range of phages as compared to the natural phage resistance mechanisms which have evolved through the never ending arms race between phages and bacteria <sup>81</sup>. They might however be used as supplements to natural anti-phage barriers to increase the overall efficiency by the combined effects of stacking different phage resistance mechanisms. A different problem using engineered phage resistance in industrial bacterial strains is the characterization of these strains as genetically modified organisms (GMO). The industrial use of GMOs is prevented by legal limitations in many countries <sup>54,107</sup> but also consumer skepticism is becoming an increasing problem for the use of GMO microorganisms <sup>33,54,153</sup>.

Despite the focused research in lactococcal phage biology and the emergence of still more sophisticated molecular tools, problems with phages in industrial fermentations remain a significant economical issue for the dairy industry. Combined with the legislative limitations as well as consumer skepticism against GMO, this explains the constant need for discovering and/or

improving naturally existing phage resistance mechanisms without using the molecular tools regarded as genetic manipulation.

### **CRISPR – a novel type of phage resistance system**

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are found in most Bacteria and Archaea<sup>4</sup>. CRISPRs are found in 50% of the known lactic acid bacteria genomes and though they have not been observed in *L. lactis* a brief description of this novel type of phage resistance mechanisms is included in this section.

A CRISPR is a locus consisting of short (ca 20 bp) conserved repeats which are interspaced by other short sequences (ca 20 bp) with a fixed length known as spacers. These spacers consist primarily of DNA originating from phages or plasmids. CRISPR associated genes (*cas*) are located adjacent to the CRISPR sequence. By analyzing the CRISPR loci of a phage resistant mutant of *S. thermophilus* charged with a specific phage, it was revealed that this mutant had added repeats and spacers, the latter matching DNA sequence from the phage against which the mutant had acquired resistance<sup>4</sup>. By cloning the newly acquired spacers in the wt strain and confirming that the strain had become resistant to the specific phage, the authors demonstrated that the phage resistance phenotype was indeed caused by the acquired extra spacer sequences. Knock-out experiments revealed that also the *cas* genes were involved in the resistance phenotype and acquisition of new spacer sequences. For the CRISPR phage resistance system to function, 100% sequence identity between spacer and phage is required. Since such a high level of sequence identity is required for the efficacy of the CRISPR system, it is not surprising that phage mutants that overcome the system have mutated primarily by single nucleotide mutations but also deletions in the genes that matches the spacer sequences in order to escape the CRISPR system<sup>37</sup>.

One of the most interesting aspects of the CRISPR phage resistance system is that the resistance is acquired by the bacterium and that it is heritable in the bacterial cell line<sup>4</sup>. It was shown that continuous charging with different phages lead to iterative addition of spacers thereby extending the range of phages against which the system provides protection<sup>37</sup>. This strongly suggest that prokaryotes have evolved a kind of a DNA based trainable immunity system in which the spacer sequences determine the range of immunity and the *cas* proteins mediate the resistance<sup>4</sup>.

# **MANUSCRIPT I**





# **AbiV, a novel abortive phage infection mechanism on the chromosome of *Lactococcus lactis* subsp. *cremoris* MG1363**

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Accepted for publication in Applied and Environmental Microbiology

## **ABSTRACT**

Insertional mutagenesis with pGhost9::ISS1 resulted in independent insertions within a 350 bp region of the chromosome of *Lactococcus lactis* subsp. *cremoris* MG1363 that conferred phage resistance to the integrants. Orientation and location of the insertions suggested that the phage resistance phenotype was caused by a chromosomal gene turned on by a promoter from the inserted construct. RT-PCR analysis confirmed higher levels of transcription of a downstream *orf* in the phage-resistant integrants compared to the phage-sensitive *L. lactis* MG1363. This gene was also found to confer phage resistance to *L. lactis* MG1363 when cloned into an expression vector. A subsequent frameshift mutation in the *orf* completely abolished the phage resistance phenotype confirming that the ORF was necessary for phage resistance. This ORF provided resistance against virulent lactococcal phages of the 936 and c2 species with efficiency of plaquing (EOP) of 10<sup>-4</sup> but did not protect against members of the P335 species. High expression of the ORF did not affect cellular growth rate. Assays for phage adsorption, DNA ejection, restriction/modification activity, plaque size, phage DNA replication, and cell survival showed that the ORF encoded an abortive infection (Abi) mechanism. Sequence analysis revealed a deduced protein of 201 amino acids,

which, in its native state, probably forms a dimer within the cytosol. Similarity searches showed no homology to other phage resistance mechanisms and thus, this novel Abi mechanism was named AbiV. The mode of action of AbiV is unknown but its activity prevented the cleavage of the replicated phage DNA of 936-like phages.

## INTRODUCTION

The use of *Lactococcus lactis* starter cultures for the production of fermented dairy products is a worldwide practice. However, bacteriophage infection of the added starter cultures may lead to fermentation delays or even halt the process. Despite considerable progress made over the past decades that has led to improve phage control measures, this natural phenomenon still remains a significant risk to the dairy industry <sup>2,14,53</sup>. Three groups of lactococcal phages cause the vast majority of milk fermentation failures, namely members of the 936 and c2 species, which display great intra-species homogeneity but are genetically distinct from each other <sup>12,29,48</sup> and phages from the P335 species which exhibit a much greater genomic mosaicism <sup>12,42</sup>. To survive this phage diversity *L. lactis* strains possess a wide variety of resistance mechanisms. Lactococcal phage defense systems are classified into four general groups depending on the step of the phage lytic cycle they inhibit <sup>37</sup>. The first two groups either prevent phage adsorption or block DNA ejection <sup>53</sup>. The last two groups are intracellular antiphage hurdles, namely restriction/modification systems and abortive infection (Abi) mechanisms, the latter are reviewed in <sup>14,70</sup> and are arguably the most efficient.

To date, twenty-two lactococcal Abi mechanisms have been isolated, characterized, and designated AbiA through AbiZ <sup>14,24</sup>. Remarkably, most of them appear to have a distinct mode of action, although they do share some common features. These include reduction in burst size, reduction in efficiency of plaquing <sup>18,25</sup>, reduction in efficiency to form centers of infection (ECOI) as well as death of the infected cells <sup>70</sup>. The Abi phenotype is usually mediated by a single gene though in a few cases (AbiE, G, L, T, and U), the system consists of two genes <sup>9,18,19,30,60</sup>. Some degree of sequence similarity exists between different Abi proteins <sup>14</sup>, but in general, they show little similarity to other genes or proteins in databases. This feature is in agreement with their antiviral activity towards specific phages or phage groups as well as their particular mechanistic models. Indeed, the characterized Abi mechanisms from wild-type *L. lactis* strains have been shown to disable members of one, two or more lactococcal phage groups, although there is a tendency towards a broader efficacy against 936 phages <sup>14</sup> which represent the group causing the most dairy fermentation failures. The general effects on the phage lytic cycle have been revealed for most Abi mechanisms, although in most cases much further characterization is still required. For example, AbiA, F, K, P, and T were shown to interfere with DNA replication <sup>9,22,26,30,34</sup> while AbiB, G, and U affected RNA transcription <sup>15,18,61</sup>. AbiC was shown to cause limited major capsid protein

production <sup>57</sup> whereas AbiE, I, and Q affected phage packaging <sup>25</sup>. AbiD1 was found to interfere with a phage RuvC-like endonuclease <sup>6,7</sup> and the presence of AbiZ caused premature lysis of the infected cells <sup>24</sup>.

Most Abi systems are encoded on plasmids of which some are conjugative, thereby enabling the lateral transfer of the resistance mechanism to phage sensitive *L. lactis* strains and the subsequent industrial use of the phage-resistant derivatives. Usually, Abi mechanisms provide a much stronger phage resistance phenotype when they are plasmid-encoded (due to higher gene copy number) as compared to chromosomally-encoded systems which may explain why so few Abi genes are encoded on bacterial genomes <sup>15,62,64</sup>.

Natural Abi mechanisms have been used extensively for protection of industrial starter cultures <sup>16,27</sup>. Not surprisingly, this added selective pressure has led to the emergence of phage mutants capable of overcoming these resistance barriers <sup>16,27</sup>. Therefore, it is highly desirable to isolate novel phage resistance mechanisms that can be used alone or in combination with other mechanisms to provide increased phage protection to the starter cultures <sup>53</sup>.

We report here a novel lactococcal Abi system, named AbiV, which is chromosomally-encoded and effective against virulent phages of the 936 and c2 species. This novel system was discovered through insertional mutagenesis of the laboratory workhorse strain *L. lactis* MG1363. Although insertional mutagenesis is usually used in loss-of-function studies to characterize the effect of a genetic knock-out <sup>23,47,50</sup>, our data indicated that the expression of AbiV was turned on by the insertion of pGhost9::ISS1. The microbiological and molecular characterizations of AbiV are also presented.

## MATERIALS AND METHODS

**Bacterial strains, phages, and plasmids.** Bacterial strains used in this study are listed in Table 1, whereas bacteriophages and plasmids are listed in Table 2. Bacteriophages sk1 and jj50 were kindly provided by F.K. Vogensen (University of Copenhagen). *L. lactis* strain MB112 was used as host for the selection of phage resistant integrants. This strain is a derivative of *L. lactis* subsp. *cremoris* MG1363, with a mutation in the *upp* gene. This mutation renders *L. lactis* MB112 resistant to fluorouracil, which provided an additional marker to confirm that the phage resistant integrants were derived from the parental strain <sup>49</sup>. *Escherichia coli* was grown at 37°C in LB medium <sup>68</sup>. *L. lactis* was grown in M17 <sup>71</sup> supplemented with 0.5 % glucose (GM17). When fluorouracil resistance was tested, *L. lactis* strains were grown in SA medium supplemented with 0.5 % glucose <sup>36</sup>. Lactococci were grown at 30°C, except strains containing the thermo-sensitive vector pGhost9::ISS1, which were grown at 28°C or 37°C to allow replication or integration, respectively of the vector. When *L. lactis* grew at temperatures above 30°C, NaCl (1%) was added to the medium <sup>39</sup>. In phage infection experiments, 10 mM CaCl<sub>2</sub> was added to plates or medium. When appropriate, antibiotics were added as follows: for *E. coli*, 100 µg ml<sup>-1</sup> of ampicillin, 10 µg ml<sup>-1</sup> of chloramphenicol, and 150 µg ml<sup>-1</sup> of erythromycin; for *L. lactis*, 3 µg ml<sup>-1</sup> of chloramphenicol, 3 µg ml<sup>-1</sup> of erythromycin, and 0.3 µg ml<sup>-1</sup> fluorouracil.

**Insertional mutagenesis with pGhost9::ISS1 and isolation of phage-resistant mutants.** Phage-resistant derivatives of *L. lactis* MB112, were obtained by insertional mutagenesis using the vector pGhost9::ISS1, essentially as described by Maguin *et al.* <sup>47</sup>. Subsequent cloning of the flanking chromosomal DNA was also performed according to Maguin *et al.* <sup>47</sup>. To ensure the isolation of phage-resistant integrants from distinct events, the mutagenesis was performed on three independent cultures of *L. lactis* MB112 containing pGhost9::ISS1. These cultures were grown overnight at 28°C in GM17 containing erythromycin. The cultures were then diluted 100-fold in GM17 and grown for 2.5 h at 28°C to attain exponential growth. The cells were incubated at 37°C for 2.5 h to allow plasmid integration. The cultures were diluted again but 10,000-fold in 50 ml GM17 supplemented with erythromycin and incubated overnight at 37°C to allow phenotypic expression. These overnight cultures were inoculated (1 %) in fresh GM17 medium with erythromycin and incubated at 37°C. When they reached exponential growth, aliquots were mixed with the virulent lactococcal phage sk1 (MOI > 1). Following 10 min incubation at 37°C, the

infected cells were plated on GM17 plates containing erythromycin, NaCl (1%), and CaCl<sub>2</sub> (10 mM) and incubated two days at 37°C. The frequency of phage-resistant mutants was calculated and one hundred of them were selected at random from the three independent cultures. The phage resistant phenotype of these mutants was verified by cross-streaking with virulent phage sk1 at 37°C. Finally, curing the phage-resistant mutants of the pGhost9::ISS1 plasmid, thereby leaving a single copy of ISS1 at the integration site, was performed as described by Maguin *et al.* <sup>47</sup>.

**RNA isolation, purification and RT-PCR analysis.** Overnight cultures were diluted 100-fold and grown to OD<sub>600</sub> of 0.5 at 37°C. Aliquots (2 ml) were harvested by quick centrifugation (20,000 g, 30 sec) and the pellet was resuspended in a solution of 0.5 M sucrose with 60 mg ml<sup>-1</sup> lysozyme. Following incubation (37°C, 15 min), the cells were pelleted and resuspended in 1 ml TRIzol Reagent (Invitrogen). Total RNA was isolated according to the manufacturer's instructions. Prior to reverse transcription (RT)-PCR, RNA samples were treated with the DNase based TURBO DNA-free kit (AB).

RT-PCR was carried out using the RevertAid First Strand cDNA Synthesis kit (Fermentas) as recommended by the manufacturer. As a control, the RT-PCR procedure was carried out without reverse transcriptase to ensure that the RNA samples were free of contaminating DNA.

**Phage assays.** Propagation of phages <sup>26</sup>, titers of phage lysates <sup>40</sup>, efficiency of plaquing (EOP) <sup>69</sup> and cross streaking assays <sup>67</sup> were performed as described previously. Adsorption assays were conducted as described elsewhere <sup>69</sup>, except that a 5 min incubation period was used instead of 15 min. Cell survival <sup>5</sup> was assayed using a multiplicity of infection (MOI) of 5. One-step growth curve experiments and center of infection (COI) assays <sup>57</sup> were performed using MOIs of 0.2 and 0.5, respectively. ECOI (efficiency of COI) and burst size was calculated as previously reported <sup>57</sup>.

**Phage DNA replication.** Replication of phage DNA was followed in a time course experiment <sup>33</sup>. Briefly, total DNA of *L. lactis* cells was isolated at 10 min intervals from cultures infected with reference phage p2 (MOI of 2), which is closely related to phage sk1. Total DNA was digested with EcoRV and heated (65°C, 10 min) prior to gel electrophoresis allowing identification of resolved *cos* sites that were used to distinguish concatemeric and mature phage DNA.

**TABLE 1.** Bacterial strains used in this study

Bacterial strain	Relevant characteristics <sup>a</sup>	Source
<b><i>L. lactis</i></b>		
IL1403	subsp. <i>lactis</i> , host for 936-like phages	8
MB112	subsp. <i>cremoris</i> MG1363, $\Delta upp$ , host for 936- and c2-like phages	49
MG1363	subsp. <i>cremoris</i> , host for 936-like and c2-like phages	31
SMQ-86	subsp. <i>cremoris</i> , host for P335-like phages, $Erm^R$	26
JH-20	MB112 (pJH2), $Cam^R$ , $Abi^+$	This study
JH-22	IL1403 (pJH2), $Cam^R$ , $Abi^+$	This study
JH-23	SMQ-86 (pJH2), $Cam^R$ , $Abi^+$	This study
JH-24	MB112 (pJH3), $Cam^R$ , $Abi^-$	This study
JH-32	MB112 (pGhost9::ISS1 inserted into Lin at 1962); $Erm^R$ , $Abi^+$	This study
JH-46	MB112 (pGhost9::ISS1 insertion into Lin at 1962); $Erm^R$ , $Abi^+$	This study
JH-47	MB112 (pGhost9::ISS1 insertion into Lin at 2296); $Erm^R$ , $Abi^+$	This study
JH-48	MB112 (pGhost9::ISS1 insertion into Lin at 2240); $Erm^R$ , $Abi^+$	This study
JH-49	JH-32 cured of pGhost9, $ISS1^+$ , $Abi^-$	This study
JH-50	JH-46 cured of pGhost9, $ISS1^+$ , $Abi^-$	This study
JH-51	JH-47 cured of pGhost9, $ISS1^+$ , $Abi^-$	This study
JH-52	JH-48 cured of pGhost9, $ISS1^+$ , $Abi^-$	This study
JH-53	MB112 (pJH6), $Cam^R$ , $Abi^-$	This study
JH-54	MB112 (pLC5), $Cam^R$ , $Abi^-$	This study
JH-79	MG1363 (pJH2), $Cam^R$ , $Abi^+$	This study
<b><i>E. coli</i></b>		
EC1000	$RepA^+$ MC1000, $Km^R$	43
JH-56	EC1000 (pJH7), $Erm^R$	This study
JH-57	EC1000 (pJH8), $Erm^R$	This study
JH-58	EC1000 (pJH9), $Erm^R$	This study
JH-59	EC1000 (pJH10), $Erm^R$	This study
JH-62	M15 (pJH11), $Km^R$ , $Amp^R$	This study
M15	pREP4, $Km^R$ , used for cloning of pQE-70 His-tag vector	Qiagen
MC1061	Laboratory strain, cloning host	52
TOP10F'	Chemically competent cells from the TOPO TA cloning kit	Invitrogen

<sup>a</sup>Lin refers to GenBank sequence accession number AF324839.  $Abi^+$ , phage resistance phenotype;  $Abi^-$ , phage sensitive phenotype;  $Amp^R$ , ampicillin resistance;  $Cam^R$ , chloramphenicol resistance;  $Erm^R$ , Erythromycin resistance;  $Km^R$ , Kanamycin resistance.



**TABLE 2.** Phages and plasmids used in this study

Phage/plasmid	Relevant characteristic <sup>a</sup>	Source <sup>a</sup>
<b>Phages</b>		
bIL170	Small isometric headed, 936 species	17
jj50	Small isometric headed, 936 species	38,48
p2	Small isometric headed, 936 species	58
P008	Small isometric headed, 936 species	44,48
sk1	Small isometric headed, 936 species	13
712	Small isometric headed, 936 species	48
c2	Prolate headed, c2 species	69
bIL67	Prolate headed, c2 species	69
eb1, ml3	Prolate headed, c2 species	58
ul36	Small isometric headed, P335 species	41,55
ul36.k1t1	Small isometric headed, P335 species	42
ø31, ø50	Small isometric headed, P335 species	1
P335	Small isometric headed, P335 species	11
Q30, Q33	Small isometric headed, P335 species	54
<b>Plasmids</b>		
pCI372	Cloning vector for <i>E. coli</i> and <i>L. lactis</i> , Cam <sup>R</sup>	32
pCR II-TOPO	Cloning vector, Amp <sup>R</sup>	Invitrogen
pGhost9::ISS1	Temperature sensitive vector used for mutagenesis, Erm <sup>R</sup>	47
pJH2	1021-2320 fragment cloned in PstI site of pLC5; Cam <sup>R</sup>	This study
pJH3	pJH2 cut in ClaI site, filled with Klenow, <i>abiV</i> frameshift mutation; Cam <sup>R</sup>	This study
pJH6	1021-2320 fragment cloned in PstI and XbaI sites of pCI372	This study
pJH7	HindIII rescue of pGhost9::ISS1 with flanking chromosomal DNA of JH-32	This study
pJH8	HindIII rescue of pGhost9::ISS1 with flanking chromosomal DNA of JH-46	This study
pJH9	HindIII rescue of pGhost9::ISS1 with flanking chromosomal DNA of JH-47	This study
pJH10	HindIII rescue of pGhost9::ISS1 with flanking chromosomal DNA of JH-48	This study
pJH11	1273-1875 fragment cloned in pQE-70, C-terminal His-tag, Amp <sup>R</sup>	This study
pLC5	<i>E. coli</i> - <i>L. lactis</i> shuttle expression vector, promoter in front of PstI, Cam <sup>R</sup>	This study
pQE-70	Cloning vector for C-terminal His-tagging of proteins, Amp <sup>R</sup>	Qiagen

<sup>a</sup> Fragment refers to GenBank accession number AF324839; Amp<sup>R</sup>, ampicillin resistance; Cam<sup>R</sup>, chloramphenicol resistance; Erm<sup>R</sup>, erythromycin resistance; Km<sup>R</sup>, kanamycin resistance.

**Phage adsorption and DNA injection assays using SYBR-Gold staining.** Visualization of phage DNA by labeling with the fluorescent dye SYBR-Gold was performed as described by Noble and Fuhrman<sup>59</sup> with the following modifications. The original SYBR-Gold solution was first diluted 1000-fold. The phage lysate was treated with 1  $\mu\text{g ml}^{-1}$  DNase and RNase for 30 min at 37°C and then stained with the diluted SYBR-Gold to give a final concentration of 2.5% (vol/vol). The mixture was incubated for a minimum of 12 h at 4°C in the dark. One  $\mu\text{l}$  of the labeled phage lysate was mixed with 1  $\mu\text{l}$  exponentially growing cell culture and visualized under a Zeiss axioplan microscope.

**Rescue cloning and sequencing of the flanking chromosomal DNA.** Chromosomal DNA was analyzed for six phage-resistant integrants. The HindIII-digested DNA was purified, diluted to favor self-ligation and ligated as described by Maguin *et al.*<sup>47</sup>. The ligation mixture was electroporated in *E. coli* EC1000<sup>43</sup> and plated on LB agar with erythromycin. Plasmid DNA containing the rescued pGhost9::ISSI and the flanking chromosomal DNA was isolated from *E. coli* cells (JH-56 to JH-59). Chromosomal DNA flanking the ISSI element was sequenced using the primer (5'-GAAGAAATGGAACGCTC-3'), annealing to the ISSI sequence. The above procedure was successful for four of the six candidates.

**DNA isolation and manipulation.** Plasmid DNA was isolated from *L. lactis* using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's recommendation with the exception that lysozyme (15  $\text{mg ml}^{-1}$ ) was added to buffer P1 and the lysis solution with the resuspended cells was incubated at 37°C for 30 min before proceeding with the protocol. Restriction enzymes, T4 DNA ligase, and Klenow fragment (Fermentas) were used according to the manufacturer's instructions. Electroporation of *L. lactis* was performed as described previously<sup>35,56</sup>. The DNA fragment corresponding to nucleotides 1021 to 2320 in the GenBank sequence AF324839 (Fig. 1) was first cloned into the *E. coli* pCR II-TOPO vector (Invitrogen) using TOP10F' cells prior to cloning into the *E. coli*-*L. lactis* shuttle vectors pCI372 and pLC5. For protein purification, the DNA fragment corresponding to nucleotides 1273 to 1875 in the GenBank sequence AF324839 was cloned into the His-tag vector pQE-70 (Qiagen) as recommended by the manufacturer to create pJH11 in the strain JH-62.

**Protein purification and gel-filtration.** A C-terminally his-tagged protein was purified according to the manufacturer's instructions (Qiagen). Gel-filtration was carried out on a HiPrepTM 16/60

Sephacryl™ S-300 High-Resolution column from GE Healthcare, using 150 µg AbiV and a flowrate of 0.2 ml min<sup>-1</sup>. The column was connected to a Shimadzu HPLC system. The solvent delivery module used was LC-10ADvp and the detector used was SPD-M10Avp (280 nm). The composition of the mobile phase was 50 mM Tris-HCl, 200 mM NaCl pH 7.6. A Bio-RAD protein standard (cat. No. 151-1901) was used to determine the molecular weight of the purified protein. Each run lasted 800 minutes. The molecular weight was estimated by taking the mean of four different trials.

**Construction of the expression vector pLC5.** The low-copy vector pGKV259<sup>73</sup> was digested with PstI (located downstream from the P<sub>59</sub> promoter) followed by gel purification. Two complementary oligonucleotides

(5'- TGGATCCAAAGGAGGTCCTGCA-3' and 5'- GGACCTCCTTTGGATCCATGCA-3')

were annealed together<sup>68</sup> to create a double-stranded linker with PstI-compatible sticky ends. This linker also contained a unique BamHI site and a ribosome-binding site (RBS: 5'-AGGAGG-3'). The linker was inserted into the PstI site of pGKV259 and the ligation mixture was transformed into *E. coli* MC1061. Transformants were selected on LB plates containing 20 µg ml<sup>-1</sup> of chloramphenicol. Positive clones with the linker inserted in the right direction were identified by colony PCR and clones were confirmed by sequencing. Upon introduction of the linker into pGKV259, the PstI site on the 5'-side of the linker was disrupted whereas the one on the 3'-side was conserved. Thus, a unique PstI site was created 8-bp downstream from the RBS. Cloning of a DNA fragment possessing an ATG start codon into the PstI site of pLC5 enables efficient transcription from the P<sub>59</sub> promoter, and translation from the introduced RBS.

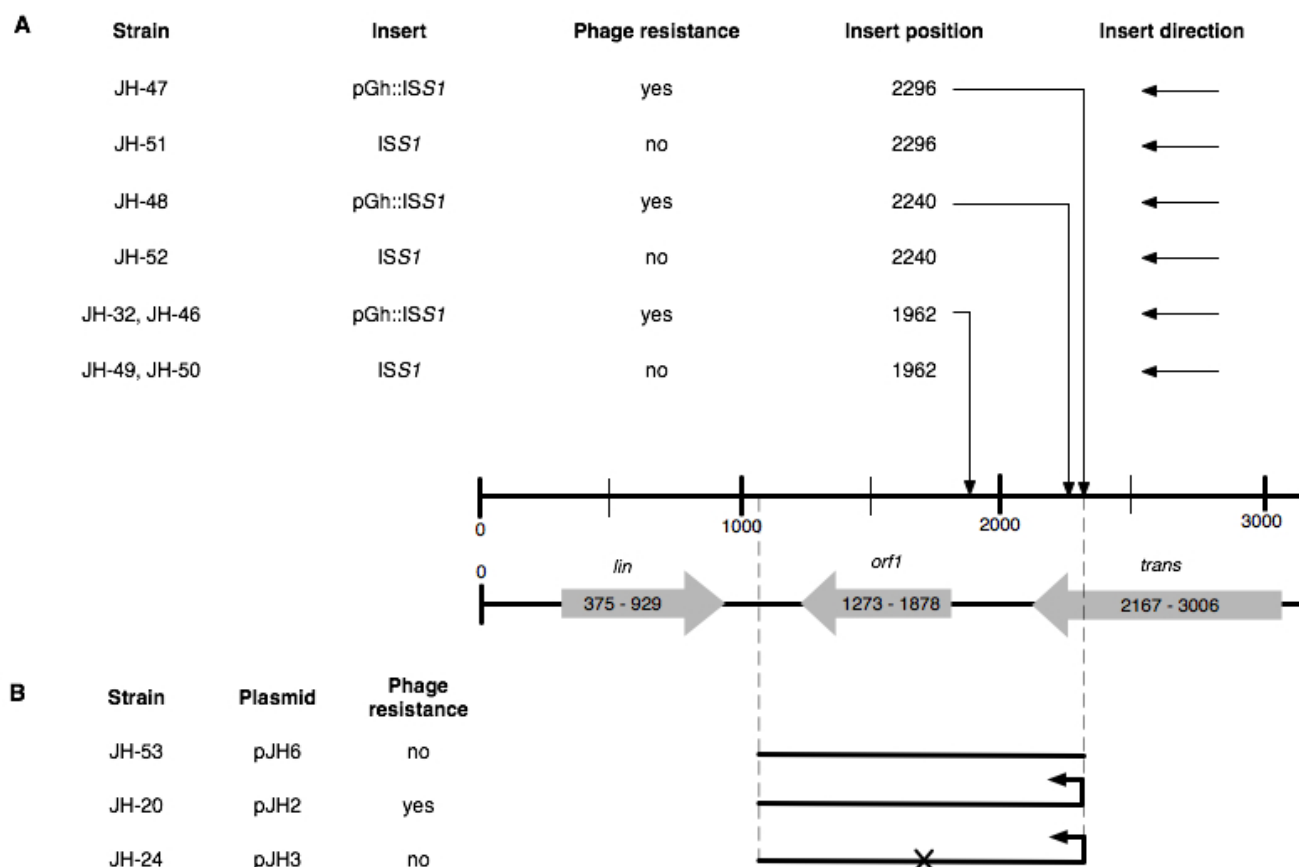
**DNA and protein analyses.** Sequence similarity searches in databases were performed using BLAST<sup>3</sup> at the NCBI website: <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>. Search for helix-turn-helix motifs were done using the website: <http://npsa-pbil.ibcp.fr/>. Molecular weight and pI of the investigated proteins were estimated using the Protein Calculator at the website: <http://www.scripps.edu/~cdputnam/protcalc.html>. Searches for transmembrane domains and signal peptide motifs were performed using the predictor websites <http://www.cbs.dtu.dk/services/TMHMM-2.0/> and <http://www.cbs.dtu.dk/services/SignalP/>.

## RESULTS

**Isolation of phage-resistant mutants of *L. lactis* subsp. *cremoris* MG1363 using insertional mutagenesis.** The laboratory strain *L. lactis* subsp. *cremoris* MB112 (MG1363 $\Delta$ *upp*)<sup>49</sup> is sensitive to several virulent phages from the 936 and c2 species. To identify genes involved in the sensitivity of *L. lactis* MG1363 to 936 phages, mutant cells resistant towards phage sk1 were isolated after insertional mutagenesis of the host. The insertional mutagenesis was performed on three cultures resulting in the isolation of three independent insertion libraries. Selection for resistance to virulent lactococcal phage sk1 was performed for all three libraries (MOI > 1). The frequency of phage resistant colonies was  $6.2 \pm 2.6 \times 10^{-6}$  which is 100-fold higher as compared to the frequency of spontaneous phage resistant mutants obtained from wild-type cultures ( $5.9 \pm 3.0 \times 10^{-8}$ ).

**Identification of a chromosomal gene involved in phage resistance.** A total of 6 confirmed phage-resistant mutants (two randomly selected from each of the three independent cultures) were chosen for plasmid rescue experiments. In four cases it was possible to isolate the inserted plasmid along with a piece of flanking chromosomal DNA (pJH7 to pJH10). Sequence analysis revealed insertions within the same 350 bp region on the chromosome of the four phage-resistant mutants. The insertions were located within the intergenic region between two genes (designated *orf1* and *trans*) or within the 3' end of the *trans* gene (Fig. 1A). The inserts were located at nucleotide 1962 (for phage resistant mutants JH-32 and JH-46), 2240 (JH-48), and 2296 (JH-47). The nucleotide positions are based on the sequence available under the GenBank accession number AF324839. JH-32 and JH-46 originated from the same mutation library and may be daughter cells. Since insertions in the three different phage-resistant mutants were observed both within the *trans* gene and in the intergenic region between *trans* and *orf1*, the phage resistance phenotype could not be caused by a knocked out *trans* gene. Furthermore, the *lin* gene downstream of *orf1* is transcribed in the opposite direction. Taken altogether, these results pointed toward *orf1* as a key player in the phage resistance phenotype.

At least two hypotheses could explain the involvement of *orf1* in the phage resistance phenotype. *Orf1* could be part of an operon transcribed from a promoter upstream of *trans* and thus terminated by the pGhost9::ISS1 insertions, thereby inactivating the transcription of the gene. A second possibility could be that the pGhost9::ISS1 insertion provided a promoter upstream of *orf1*. In this latter scenario, the transcription of *orf1* would be initiated from a promoter within the ISS1

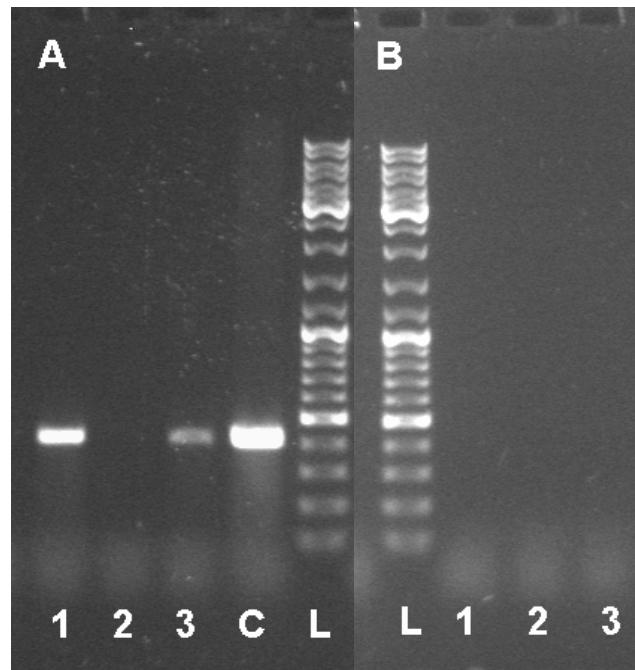


**Fig. 1.** Localization of *orf1* (*abiV*) on the chromosome of *L. lactis* MG1363. Numbers refer to nucleotide positions in the GenBank sequence AF324839. (A) *L. lactis* strains with the transposon containing vector pGhost9::ISS1 or cured of pGhost9 thus containing a solitary ISS1 inserted on the chromosome. Numbers and arrows indicate the position and direction of the inserts. (B) *L. lactis* strains with the cloned DNA fragment (bp 1021-2320) including *orf1*. Arrows indicate promoter P<sub>59</sub> and the × in JH-24 represents the position of the frameshift mutation introduced into this strain.

sequence or from the Em<sup>r</sup> gene in the pGhost9::ISS1 construct, leading to the activation of a phage resistance phenotype. When the insertion mutants were cured of the vector pGhost9::ISS1, leaving a single copy of ISS1 at the integration site, the cured *L. lactis* strains (JH-49 to JH-52, Table 1) lost the phage resistance phenotype, suggesting that a promoter activity originated from the vector, possibly from the Em<sup>r</sup> gene (Fig. 1A).

To test the hypothesis that *orf1* was transcribed from a promoter within the inserted pGhost9::ISS1, a RT-PCR assay was made on *L. lactis* strains with and without the insertion. RNA from exponentially growing cells was used as template for random reverse transcription of total RNA and primers annealing to an internal region of *orf1* were used to amplify the cDNA by PCR. A PCR product of the expected size was observed in the mutant with the insertion (Fig. 2, Lane 3) but not in the wild-type strain (Fig. 2, Lane 2), indicating that the transcription of *orf1* was turned on in the mutant. A negative control experiment was conducted without reverse transcriptase confirming that the samples were free of contaminating DNA (Fig. 2B). A positive control

experiment was included using primers for the glycolytic genes *gapB* and *pfk* (data not shown). The positive result of this experiment excluded artifacts due to loss of RNA in the sample.



**Fig. 2.** RT-PCR (20 PCR cycles) on RNA isolated from exponentially growing *L. lactis* cultures. Lane 1, JH-20 (pJH2); Lane 2, JH-54 (pLC5); Lane 3, JH-32 (insertion mutant); Lane C, control PCR with *L. lactis* MB112 chromosomal DNA; Lane L, ladder (GeneRuler™, 100-10,000 bp (Fermentas)). (A) The experiment was performed with reverse transcriptase. (B) The experiment was performed without reverse transcriptase.

**A silent phage resistance mechanism encoded by *orf1* exists on the chromosome of *L. lactis* subsp. *cremoris* MG1363.** To test the hypothesis that *orf1* is encoding a phage resistance mechanism, a DNA fragment (positions 1021 to 2320 in the GenBank sequence AF324839) comprising only one ORF (*orf1*) and the upstream region including the most distant insertional mutation site at position 2296 was cloned in the promoter-less shuttle vector pCI372 (pJH6) and in the expression vector pLC5 (pJH2) (Fig. 1B). These constructs were transformed in *L. lactis* MB112 and the resulting strains (JH-53 and JH-20, respectively) were tested for phage resistance. *L. lactis* JH-53 (pJH6, with no promoter upstream of *orf1*) was sensitive to phage sk1 whereas *L. lactis* JH-20 (pJH2, with a strong promoter upstream of *orf1*) was resistant to phage sk1 (Fig. 1B). In fact, the highest level of expression was observed when *orf1* was expressed from the pLC5 vector, which was probably due to the strong P<sub>59</sub> promoter (Fig. 2, lane 1 and 3). Furthermore,

expression of *orf1* (pJH2) in another host (*Lactococcus lactis* subsp. *lactis* IL1403) also conferred a phage resistance phenotype. The above results indicated that the expression of *orf1* was responsible for the phage resistance phenotype.

To verify that a protein encoded by *orf1* was responsible for the resistance to phage sk1, a frameshift mutation was introduced into *orf1*, by digesting at a unique ClaI site (codon 36 in *orf1*), filling the ends with Klenow fragment followed by ligation, confirmation by sequencing, and transformation of the resulting plasmid pJH3 into *L. lactis* MB112. The frameshift mutation abolished the phage resistance phenotype in the resulting strain JH-24 (Fig. 1B). We concluded that the protein encoded by *orf1* was responsible for the phage resistance phenotype.

**Sequence and analysis of *orf1*.** The 1300 nucleotide DNA fragment cloned into pJH2 was sequenced and found to be 100 % identical to the region encompassing nucleotides 1021 to 2320 in the GenBank accession number AF324839 as well as positions 697547 to 698846 in the complete genome sequence of *L. lactis* MG1363 (AM406671). The G+C content of *orf1* was 31.7 %. No suitable promoter could be found upstream of *orf1* (bp 1 to 430) using Winseq software (F. G. Hansen, unpublished). This is in agreement with the experimental results that *orf1* is silent in wild-type *L. lactis* MG1363. The translation start codon of *orf1* was preceded by a suitable ribosome-binding site (5'-TGAACGGAGAG-3', underlined sequence matches consensus sequence). DNA sequence analysis did not identify any transcription terminator structures between *orf1* and the upstream *trans* gene leaving the possibility that *orf1* could be part of an operon initiated upstream of *trans*. However, the negative RT-PCR data for *orf1* transcription in the wild type strain (Fig. 2, Lane 2) suggests that *orf1*, and perhaps *trans* as well, is not expressed under the conditions tested. This was further confirmed by Northern analysis of the insertion mutant *L. lactis* JH-32 and wild-type *L. lactis* MG1363 in which transcription of *orf1* was observed only in JH-32 (data not shown).

**The phage resistance mechanism encoded by *orf1* is effective against virulent lactococcal phages of the 936 and c2 species.** Representatives from the three main lactococcal phage species, namely 936, c2, and P335, were tested for their sensitivity to *orf1* (Table 3). Efficiency of plaquing (EOP) values of approximately  $10^{-4}$  were obtained for the four tested c2-like phages and for five of the six tested members of the 936 species (Table 3). Phage 712 (936 species) was insensitive to *orf1* (EOP of 1, Table 3). The seven phages of the P335 species were also unaffected by the presence of *orf1* (Table 3). Because the efficacy of *orf1* on virulent P335 phages was tested with another *L.*

*lactis* host (SMQ-86), we needed to rule out the possibility that the insensitivity of P335 phages was due to a mutation in pJH2 present in *L. lactis* SMQ-86 (*L. lactis* JH-23). Thus, the plasmid pJH2 was isolated from *L. lactis* JH-23 and re-introduced into *L. lactis* MB-112. Phage p2 was unable to effectively replicate on this recombinant host (EOP of  $10^{-4}$ ), indicating that pJH2 was indeed functional and confirming that the P335 phages are insensitive to *orf1*.

**TABLE 3.** Efficiency of plaquing (EOP)

	Phage	<i>L. lactis</i> strain	EOP <sup>c</sup>
936 species <sup>a</sup>	sk1	JH-20 (pJH2)	$2.7 \pm 1.4 \times 10^{-4}$
	p2	JH-20 (pJH2)	$4.8 \pm 1.8 \times 10^{-4}$
	jj50	JH-20 (pJH2)	$8.3 \pm 0.5 \times 10^{-5}$
	712	JH-20 (pJH2)	$1.1 \pm 0.2$
	P008	JH-22 (pJH2)	$3.8 \pm 1.5 \times 10^{-4}$
	bIL170	JH-22 (pJH2)	$3.1 \pm 1.2 \times 10^{-4}$
c2 species <sup>a</sup>	c2	JH-20 (pJH2)	$5.2 \pm 0.4 \times 10^{-5}$
	bIL67	JH-20 (pJH2)	$2.0 \pm 1.2 \times 10^{-4}$
	ml3	JH-20 (pJH2)	$3.4 \pm 0.3 \times 10^{-4}$
	eb1	JH-20 (pJH2)	$2.2 \pm 0.7 \times 10^{-4}$
P335 species <sup>b</sup>	ul36	JH-23 (pJH2)	1.0
	ul36.k1t1	JH-23 (pJH2)	1.6
	ø31	JH-23 (pJH2)	1.0
	ø50	JH-23 (pJH2)	1.0
	Q33	JH-23 (pJH2)	0.7
	Q30	JH-23 (pJH2)	0.8
	P335	JH-23 (pJH2)	0.4

<sup>a</sup> EOP of 936- and c2-like phages is 1.0 on *L. lactis* MG1363 (MB112) and MB112 + pLC5 (JH-54). EOP of phages P008 and bIL170 is 1.0 on *L. lactis* IL1403.

<sup>b</sup> EOP of P335 species is 1.0 on *L. lactis* SMQ-86.

<sup>c</sup> EOP was calculated as an average of three independent assays.

The efficiency of plaquing of 936 and c2-like phages was also tested at 30°C and 37°C and found to be similar, indicating that the system is stable within this temperature range. EOP values of  $1.6 \pm 0.2 \times 10^{-5}$  and  $2.7 \pm 0.3 \times 10^{-4}$  were obtained for phages sk1 and p2, respectively when *orf1* was expressed from the chromosome in the insertion mutants. These values are in the same range as when *orf1* is expressed from a strong promoter in the low-copy expression vector pLC5. However,



when the insertion mutants were tested against phage c2, EOP values of  $4.6 \pm 0.9 \times 10^{-1}$  were obtained, indicating that *orf1* needs higher expression to be efficient against c2. Finally, pJH2 was transformed into *L. lactis* MG1363 (JH-79) and tested for efficiency of plaquing against phages p2, 712, and c2. EOP values similar to the ones obtained on MB112 excluded the possibility that the *upp* deletion in MB112 is influencing the phage resistant phenotype.

The efficiency of *orf1* as a phage resistance mechanism was further characterized using phage p2 and *L. lactis* strains JH-20 (pJH2) and MB112 (Table 4). The ECOI of phage p2 on *L. lactis* JH-20 was  $0.5 \pm 0.2$  % indicating that only 5 out of 1000 infected cells released at least one virulent phage. One-step growth curve experiments were then performed in presence or absence of pJH2 (*orf1*). The burst size was reduced by 72 % (from  $38.8 \pm 5.7$  in MB112 to  $11.1 \pm 5.2$  in JH-20) (Table 4).

**TABLE 4.** Effect of AbiV on phage p2

Assay <sup>a</sup>	MB112 (wt)	JH-20 (AbiV)
EOP <sup>b</sup>	1.0	$4.8 \pm 1.8 \times 10^{-4}$
ECOI (%) <sup>c</sup>	100	$0.5 \pm 0.2$
Burst size (pfu/cell) <sup>d</sup>	$38.8 \pm 5.7$	$11.1 \pm 5.2$
Latency time (min) <sup>d</sup>	20-30	20-30
Cell survival <sup>e</sup> (%)	$6.1 \pm 1.3 \times 10^{-5}$	$3.1 \pm 0.3 \times 10^{-6}$
DNA replication <sup>f</sup>	+	+ (concatemeric)
Plaque size (mm)	1.5 to 1.7	pinpoint to 0.7

<sup>a</sup> All assays were conducted at 30 °C.

<sup>b</sup> n=3, EOP of phage p2 is 1.0 on *L. lactis* MG1363 (MB112).

<sup>c</sup> MOI = 0.5, n=3, ECOI of phage p2 is 1.0 on *L. lactis* MB112.

<sup>d</sup> MOI = 0.2, n=3. <sup>e</sup> MOI = 5, n=3. <sup>f</sup> MOI = 2, n=1.

**The phage resistance system encoded by *orf1* is an abortive infection (Abi) mechanism.** A series of experiments were conducted to determine the type of phage resistance mechanism encoded by *orf1*. Adsorption assays showed that the level of adsorption of phage sk1 to phage resistant cells was  $95.9 \pm 10.6$  %, which is similar to adsorption level to the wild type host. Furthermore, fluorescently labeled (SYBR-Gold) phage DNA was visualized after sk1 infection of the phage-resistant strain *L. lactis* JH-20 and the phage-sensitive strain MB112. Immediately following phage infection, a fluorescent halo of adsorbed phages was seen surrounding the host cells (data not shown). Less than 15 min after the beginning of phage infection, the fluorescent signal on the cell

surface was reduced while a bright fluorescent signal was observed in the center of the cell, indicating that the phage DNA had been ejected into the host cell (data not shown). Identical results were obtained for phage resistant and phage sensitive strains. The above results indicated that the phage resistance mechanism was not an adsorption or ejection blocking mechanism.

Smaller phage plaques can be observed at a frequency of  $10^{-4}$  on *L. lactis* JH-20, which contains *orf1*. These plaques were propagated on *L. lactis* JH-20 cells as well as on the phage-sensitive host *L. lactis* MG1363 and found to be insensitive to the antiphage mechanism, which demonstrates that it is not a restriction/modification system. A cell survival assay showed virtually no survival of cells from the sensitive or the phage resistant strain upon phage infection (Table 4). Moreover, the size of the few plaques of phage p2 arising at a frequency of  $10^{-4}$  were smaller on the phage resistant strain as compared to the wild-type sensitive *L. lactis* strain (Table 4). The above results are well-documented characteristics of abortive phage infection mechanisms. The low G+C content of *orf1* (31.7 %) is also typical of Abi mechanisms. Taken altogether, the phage resistance mechanism encoded by *orf1* is an abortive infection mechanism. Accordingly, the gene was named *abiV* and the Abi mechanism was named AbiV.

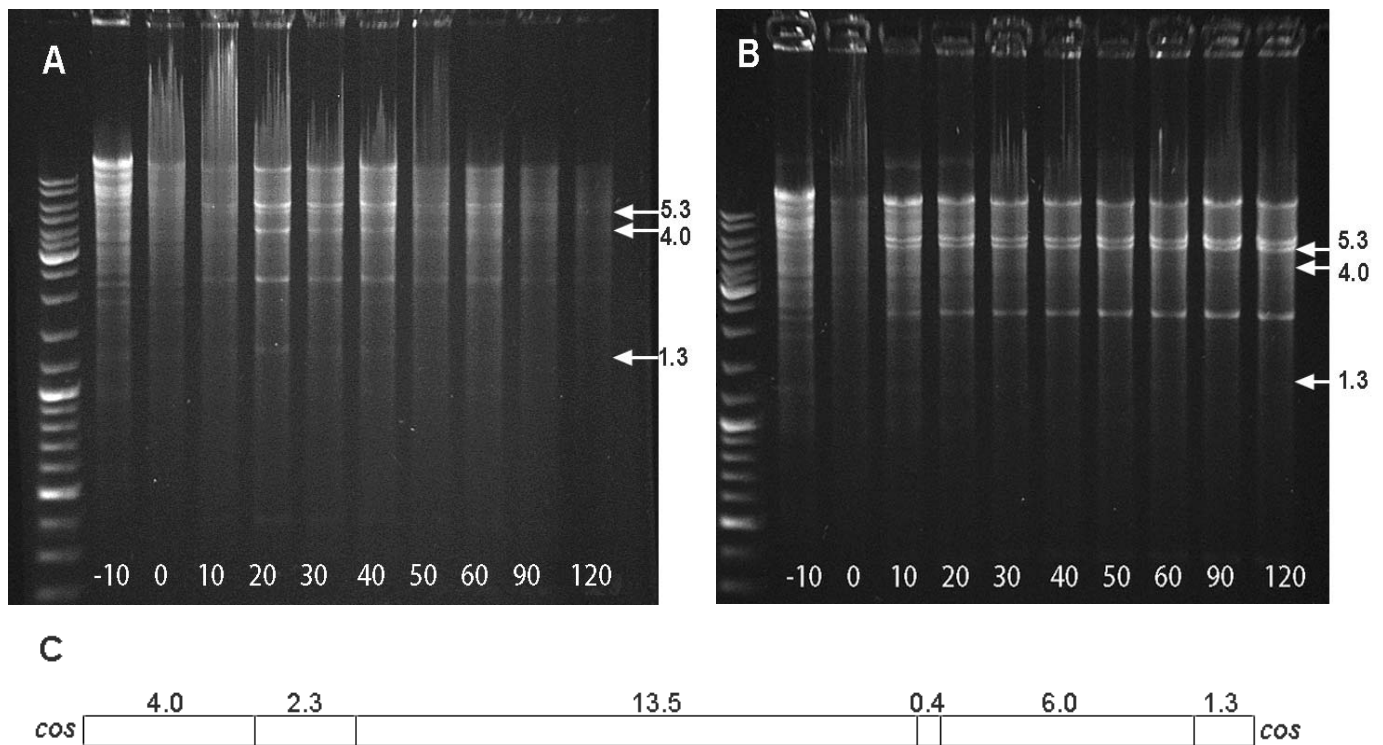
**Analysis of the AbiV protein.** AbiV consists of 201 amino acids and has a calculated molecular weight of 22,692 Da. A His-tagged AbiV protein was overexpressed in *E. coli* and purified. The native molecular weight of the purified AbiV protein was estimated to  $49 \pm 0.3$  kDa by gel filtration (data not shown), suggesting that AbiV forms a dimer in its native form. The pI was calculated to be 5.37. The protein does not contain any putative transmembrane or signal peptide motifs and it is therefore likely cytosolic. Although 69 % of AbiV consists of  $\alpha$ -helices, no helix-turn-helix motif was found in AbiV. Similarity searches using several bioinformatics tools did not reveal any similarity to other lactococcal proteins or any other phage resistance mechanism, nor was AbiV found in other sequenced lactococcal strains. Likewise, no conserved domains were found in the protein.

**Expression of AbiV does not affect cellular growth rate or final biomass.** A cell growth experiment was conducted to test whether the expression of AbiV from a strong plasmid-encoded promoter could influence the cell growth rate or final biomass. *L. lactis* JH-20 (Abi<sup>+</sup>) and MB112 (Abi<sup>-</sup>) grew exponentially in GM17 at 30°C with growth rates of  $1.04 \pm 0.08$  h<sup>-1</sup> and  $1.00 \pm 0.03$  h<sup>-1</sup>, respectively. Final biomasses were  $2.49 \pm 0.10 \times 10^9$  cells ml<sup>-1</sup> and  $2.45 \pm 0.02 \times 10^9$  cells ml<sup>-1</sup>,

respectively. From these experiments, it was concluded that the expression of AbiV did not affect cellular growth. Moreover, it showed that the newly constructed expression vector pLC5 did not interfere with cell growth.

**AbiV affects phage DNA maturation.** The DNA replication of the *cos*-type virulent lactococcal phage p2 was followed at time intervals during infection of a resistant (JH-20) and a sensitive (JH-54) *L. lactis* strain. Phage DNA was analyzed by digesting the total DNA isolated from infected cells with EcoRV and then comparing the resulting fragments with the EcoRV restriction map of phage p2 (Fig. 3). Ten minutes after infection, replication of phage DNA was observed in both strains. In sensitive cells, the concentration of phage DNA decreased around 40 min after infection coinciding with lysis of the host culture. In comparison, phage DNA persisted in the resistant cells throughout the experiment, which was terminated after 2h.

Analysis of the EcoRV-digested phage DNA pattern revealed, among others, two bands of 1.3- and 4-kb as well as a 5.3-kb fragment in the phage sensitive culture. The 5.3-kb DNA fragment spans the *cos* site on the phage p2 genome with the 1.3- and 4-kb fragments representing the mature encapsidated phage DNA. Therefore, both replicative and encapsidated DNA was observed in the phage sensitive strain due to continuous DNA replication throughout the phage life cycle and simultaneous encapsidation of mature DNA. In the resistant cells, only the 5.3-kb fragment was observed. The absence of the 1.3- and 4-kb bands as well as the presence of phage DNA throughout the experiment indicated that phage DNA accumulated in its concatemeric (non-mature) form in the resistant *L. lactis* cells. Similar results were obtained with the closely related phage sk1 (data not shown). The above results showed that AbiV prevented the cleavage of the replicated phage DNA and thus that it acts at a later stage of the phage infection process.



**Fig. 3.** Replication of phage p2 DNA during infection of the phage sensitive strain *L. lactis* JH-54 (A) and the phage resistant strain *L. lactis* JH-20 (B). Samples were taken at: -10, 0, 10, 20, 30, 40, 50, 60, 90, and 120 minutes and total DNA was restricted with EcoRV. Band 5.3 kb (consisting of bands 1.3 kb and 4 kb in mature resolved DNA) is spanning the *cos* site which marks the extremities of the phage genome. Numbers refer to the size of phage p2 DNA fragments after EcoRV digestion and heat treatment (C). Ladder is GeneRuler™, 100-10,000 bp (Fermentas).

## DISCUSSION

Insertional mutagenesis was used to confer phage resistance to *L. lactis* subsp. *cremoris* MG1363. Four phage-resistant mutants obtained from three independent insertional mutagenesis experiments were selected for further analysis and in each case the insertion was located within the same 350 bp region of the bacterial chromosome. Insertional mutagenesis with the pGhost9::ISSI system<sup>46,47</sup> was previously used to knock-out factors involved in phage adsorption<sup>23</sup> and in DNA ejection<sup>45</sup>. Such loss-of-function effects of insertional mutagenesis is the usual outcome of these experiments<sup>23,65,72</sup>. However, using a similar strategy, Luccini *et al.* also isolated a phage-resistant mutant of *Streptococcus thermophilus* in which promoter activity from the insertion turned on a downstream R/M mechanism<sup>45</sup>. A similar finding was obtained in this study as the integration of pGhost9::ISSI conferred phage resistance to the cell by activating an abortive infection mechanism.

All the ISSI inserts were oriented in the same direction as *orfI* which made us speculate that *orfI* could be transcribed from a promoter within ISSI or from the Em<sup>r</sup> gene in pGhost9::ISSI<sup>47</sup>. Promoter activity has previously been reported outward of insertion sequences<sup>28</sup> both in forward<sup>23</sup> and backward direction<sup>15</sup> and insertion sequences with a high degree of similarity to ISSI have also been known to be in the vicinity of abortive infection mechanisms<sup>4,15</sup>. Searches for promoter sequences in the forward direction of ISSI were unsuccessful. This observation was supported by the reversion to a phage sensitive phenotype when the mutants were cured of the plasmid while keeping a single copy of ISSI at the integration site (Fig. 1A). However, the activation hypothesis was supported by subsequent transcription analyses using RT-PCR on mRNA from *L. lactis* strains with and without the insertion. A higher level of transcription of *orfI* was observed in the presence of an upstream pGhost9::ISSI insertion. These results indicated that *orfI* was indeed turned on by a promoter in pGhost9::ISSI, which was most likely the promoter of the Em<sup>r</sup> gene. The cloning of *orfI* and its upstream region into the promoter-less vector pCI372 failed to confer resistance while a similar cloning into the expression vector pLC5 gave a phage resistance phenotype. An active *orfI* was later proved to encode the abortive infection protein AbiV thereby confirming the use of integration by pGhost9::ISSI to transcribe silent genes on bacterial chromosomes.

The ISSI insertion sequence is reported to integrate randomly on the chromosome of *L. lactis*<sup>47</sup>. In the present study, ISSI integrated in a 350 bp region in three independently obtained mutants conferring phage resistance. In a similar study with *L. lactis* subsp. *cremoris* Wg2 and *L. lactis* subsp. *lactis* IL1403, Dupont *et al.*<sup>23</sup> obtained phage resistant mutants that were due to a defect in

phage adsorption with frequencies around  $5 \times 10^{-6}$ , the same frequency obtained in the present study. We did not obtain any mutants with reduced phage adsorption, which indicates either that the phage receptors on *L. lactis* MG1363 are more difficult to mutate, perhaps being essential to cell growth, or that *ISSI* integrates in a non-random manner in this strain.

*abiV* is located on a 59-kb DNA region of the *L. lactis* MG1363 chromosome (nt 657,000 to 706,000) which was previously referred as an “integration hot spot”<sup>74</sup> because it contains DNA sequences involved in DNA mobility. For example, it contains genes and sequences usually found on plasmids as well as almost 20 % of the 71 insertion sequences found in the 2,529,478-bp genome of MG1363. Interestingly, two of these insertion sequences, namely *IS946* and *IS1297* share 98 % and 85 % nucleotide similarity with *ISSI*, respectively. These two IS elements exist only in one copy in the genome of *L. lactis* MG1363. The high frequency of IS elements in this region suggests that it contains features favoring the integration of insertion sequences in general and in particular of the *ISSI*-type. Thus, the *ISSI* integration may not be completely random in *L. lactis* MG1363. The location of *abiV* in the integration hot spot region among remnants of plasmid DNA and insertion sequences also makes it tempting to speculate that *abiV* originated from a plasmid. This would correspond to the observation that most of the previously described lactococcal Abi systems are encoded on plasmids<sup>2,14,27,70</sup>.

*abiV* conferred phage resistance when cloned in an expression vector but lost this function upon introduction of a frameshift mutation in the gene. Cells expressing AbiV exhibited typical abortive infection characteristics upon phage infection such as normal phage adsorption and DNA ejection, no R/M activity, and cell death. Very few infected cells released progeny phages and the burst size was also reduced. Since database searches revealed no similarity to any other phage resistance mechanism, it was concluded that AbiV is a novel Abi mechanism.

AbiV inhibits proliferation of small isometric phages from the 936 species and of prolate phages from the c2 species but it has no effect on small isometric phages from the P335 species. This range of efficacy against the three main phage groups has been observed with other lactococcal Abi mechanisms<sup>4,14,25,51,63,64</sup> but the lack of similarity with other Abi proteins suggests a different mode of action. Combinations of diverse phage resistance mechanisms are often observed on plasmids isolated from wild-type strains of *L. lactis* that are highly resistant to phages<sup>2,20,24,30</sup>. Since AbiV is a novel Abi mechanism, it may be suitable to use it in combination with other phage resistance mechanisms to confer efficient phage resistance to industrial strains of *L. lactis*.

Lactococcal Abi mechanisms are often further characterized by identifying the general step of the phage lytic cycle that is inhibited <sup>14</sup>. In infected AbiV-containing cells, we observed that phage DNA is replicated but maturation is halted resulting in the accumulation of concatemeric phage DNA. Replication of phage DNA indicated that transcription and translation of early phage genes was taking place in the presence of AbiV.

It was also observed that the efficiency of AbiV (measured as EOP) against 936 phages was not improved by expressing *abiV* from the strong lactococcal promoter P<sub>59</sub> <sup>73</sup> in a low-copy number plasmid <sup>66</sup> as compared to expression from an internal promoter in pGhost9::ISS1 and in a single copy from the chromosome. This suggests that AbiV is only needed in small amounts or that competitive inhibition of a substrate is not part of its mode of action. However, the expression level was important for the efficiency against c2 phages. Toxicity of Abi proteins has been demonstrated for AbiD1, AbiK, AbiN, and AbiO <sup>14</sup> and in at least two cases, it was associated with regulation of the Abi gene expression <sup>4,26</sup>. The absence of effects on cellular growth rate during high expression of *abiV* demonstrates that AbiV is not toxic to the cell. Absence of sequence similarity between Abi proteins <sup>10,14</sup> makes it desirable to obtain structural data on Abi proteins to investigate possible correlations between structure and function. The native multimer state of a protein might be a basic parameter to compare Abi proteins. However, except from motifs in AbiA which are putative multimerization sites <sup>21</sup>, AbiV is the only Abi mechanism for which the native state (dimer) has been determined.

In conclusion, AbiV is a novel abortive infection mechanism, which was discovered on the chromosome of *L. lactis* subsp. *cremoris* MG1363. This discovery was made possible due to transcription from a promoter within pGhost9::ISS1, which was integrated in the upstream region. AbiV is effective against 936 and c2 phages but the mode of action of this 23<sup>rd</sup> lactococcal Abi system still needs to be further investigated as it may involve an early-transcribed phage gene or gene product.

## ACKNOWLEDGMENTS

This work was funded in part by a graduate scholarship from the Technical University of Denmark to JH and a strategic grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada to SM.

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# **MANUSCRIPT II**



# Identification and characterization of a phage gene *sav* involved in sensitivity to the lactococcal abortive infection mechanism AbiV

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Submitted to Applied and Environmental Microbiology

## ABSTRACT

*Lactococcus lactis* phage mutants that are insensitive to the recently characterized abortive infection mechanism AbiV were isolated and analyzed in an effort to elucidate factors involved in the sensitivity to AbiV. Whole genome sequencing of the phage mutant p2.1 suggested that an early transcribed *orf* was responsible for AbiV sensitivity. Sequencing of the homologous region in the genome of other AbiV insensitive mutants derived from 6 other lactococcal wild-type phages revealed point mutations in the same homologous *orf*. The *orf* was named *sav* (for sensitivity to AbiV) and the encoded polypeptide was named SaV. Purification of a His-tagged SaV polypeptide by gel-filtration suggested that it formed a dimer in its native form. Moreover, overexpression of SaV in *Lactococcus lactis* and *Escherichia coli* led to a rapid toxic effect. A conserved region in SaV which is evolutionary related among different phage groups is likely responsible for the AbiV-sensitivity phenotype and the toxicity.



## INTRODUCTION

Milk fermentation failure due to bacteriophage (phage) attack of bacterial starter cultures remain a significant risk in the dairy industry <sup>1,14</sup>. Several cheese making processes, which rely on mesophilic cultures containing *L. lactis* strains to drive the fermentation, are particularly at risk for phage attacks <sup>6,47</sup>. Despite the diversity of the lactococcal phage population <sup>22</sup>, members of only three genetically distinct phage groups (936, c2 and P335) are responsible for the majority of unsuccessful fermentations <sup>45</sup>. To defend against these phages, lactococci possess a wide variety of anti-phage barriers and more than 50 phage resistance systems have already been characterized in *L. lactis* <sup>49</sup>. These anti-phage hurdles are grouped into four general mechanisms of action, among which abortive infection (Abi) systems <sup>14,65</sup> are considered to be the most efficient. To date, 23 Abi mechanisms have been characterized in *L. lactis* <sup>14,33</sup>. The Abi phenotype is often conferred by a single gene but in few cases multiple genes are needed <sup>7,19,53,71</sup>. Overall, Abi systems constitute a heterogeneous group of proteins that share limited amino acid identity <sup>36</sup>, but act after phage DNA entry and before the release of progeny phages. The consequence of the Abi activity is not only the inhibition of the phage infection but also the death of the infected cell, presumably due to phage induced deleterious effects on critical cellular functions <sup>66</sup>.

The extensive industrial use of lactococci containing phage resistance systems has led to the emergence of phage mutants, which are insensitive to the applied anti-phage barriers. This evolutionary process generates a need to constantly find new and/or improve existing phage resistance systems in order to keep phage population under control in these man-made ecological niches <sup>17,45</sup>. To improve the efficiency of an Abi system it is crucial to know the molecular mechanism by which the system interacts with its target phages. For most of the characterized lactococcal Abi systems, only general effects on the phage life cycle have been revealed. For example, AbiA, F, K, P, and T were shown to interfere with DNA replication <sup>7,25,29,32,34</sup> while AbiB, G, and U affected RNA transcription <sup>16,19,54</sup>. AbiC caused limited major capsid protein production <sup>26</sup> whereas AbiE, I, and Q affected phage packaging <sup>28</sup>. Recently, it was demonstrated that the presence of AbiZ caused premature lysis of the infected cells <sup>27</sup>. Based on the genetic diversity of the lactococcal Abi systems their modes of action are likely to be diverse. Moreover, it has been inferred that their activity may be modulated depending on the targeted lactococcal phage species <sup>5,10,14</sup>.

In order to reveal the individual mechanistic model responsible for the resistance phenotype it is necessary to determine the molecular basis of the mode of action of an Abi system against various phages. From a practical standpoint, the analysis of Abi-insensitive phage mutants offers a great opportunity to identify the targeted phage component(s). Using this approach, genes involved in the Abi sensitivity of lactococcal P335-like phages have been identified either by specific point mutations in the genome of phage escape mutants or by exchange of genetic material with a prophage<sup>7-9,23,24,40,46</sup>. In the case of virulent 936 phages, Abi sensitivity genes were identified by point mutations in the escape phage mutants<sup>4,5,7,24,30</sup> or by recombination between two virulent phages<sup>25</sup>.

Recently, we described the isolation and characterization of the lactococcal abortive infection system AbiV<sup>33</sup>. Here, we report the identification of a specific gene in the genome of phages of the 936-species and c2-species which is involved in the sensitivity to AbiV. Moreover, the phage gene product has a strong anti-microbial effect when expressed in *L. lactis* and *E. coli*. This gene appears to have an evolutionary relationship with a wide variety of phages.

## MATERIALS AND METHODS

**Bacterial strains, phages, plasmids and growth conditions.** Bacterial strains, phages and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37°C in LB medium <sup>61</sup>. *L. lactis* was grown in M17 <sup>68</sup> supplemented with 0.5 % glucose (GM17). *L. lactis* strains were grown at 30°C except for strains with pGhost9::ISSI insertions that were grown at 37°C to maintain the insertion. In phage infection experiments, 10 mM CaCl<sub>2</sub> were added to plates or medium. When appropriate, antibiotics were added as follows: for *E. coli*, 100 µg ml<sup>-1</sup> of ampicillin, 10 µg ml<sup>-1</sup> of chloramphenicol, 25 µg ml<sup>-1</sup> of kanamycin and 10 µg ml<sup>-1</sup> of tetracycline; for *L. lactis*, 5 µg ml<sup>-1</sup> of chloramphenicol, 3 µg ml<sup>-1</sup> of erythromycin, and 5 µg ml<sup>-1</sup> of tetracycline. Phage mutants insensitive to AbiV were isolated from single plaques obtained on a host expressing AbiV followed by propagation in liquid medium on the same host. The latter procedure was repeated three times for all isolated phage mutants to ensure a pure isolate.

**Phage assays.** Propagation of phages was performed as described by Emond *et al.* <sup>29</sup>. Titers of phage lysates <sup>39</sup>, efficiency of plaquing (EOP) <sup>62</sup> and cross streaking assays <sup>60</sup> were performed as described previously. One-step growth curve experiments and center of infection (COI) assays <sup>48</sup> were performed using MOIs of 0.2 and 0.5, respectively. ECOI (efficiency of COI) and burst size was calculated as reported elsewhere <sup>48</sup>.

**DNA isolation and manipulations.** Phage DNA was isolated from phage lysates (titers of minimum 10<sup>9</sup> pfu ml<sup>-1</sup>) using the Lambda Maxi Kit (Qiagen) with the following modification to the manufacturer's recommendation: Proteinase K (20 mg ml<sup>-1</sup>) was added to the cells resuspended in buffer L3 and incubated (65°C, 30 min) before addition of buffer L4. Plasmid DNA was isolated from *E. coli* cells using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's recommendation and from *L. lactis* cells with the exception that lysozyme (15 mg ml<sup>-1</sup>) was added to buffer P1 and the lysis solution with the resuspended cells was incubated at 37°C for 30 min before proceeding with the protocol. Restriction enzymes and T4 DNA ligase (Roche) were used according to the manufacturer's instructions. Electroporation of *L. lactis* was performed as described previously <sup>35,46</sup>.

**Sequencing.** Phage genome sequencing was performed with an ABI prism 3700 apparatus from the genomic platform at the research center of the Centre Hospitalier de l'Université Laval. Oligonucleotides previously used for sequencing other phage genomes <sup>43</sup> were used to sequence the major part of the phage p2.1 genome. Other oligonucleotides were designed to complete the genome sequencing of the phage mutants. Sequence data was assembled using the Staden Pregap4 version 1.5 and Vector NTI version 10.3.0 (Invitrogen).

**DNA and protein analyses.** Sequence similarity searches were performed using BLAST <sup>2</sup> and PSI-BLAST <sup>3</sup> (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Searches for protein composition of helix, strand, and sheet as well as helix-turn-helix motifs were done using the website: <http://npsa-pbil.ibcp.fr/>. RNA secondary structure and calculation of free energy was performed using the website: <http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi>. Molecular weights of the investigated proteins were estimated using Protein Calculator (<http://www.scripps.edu/~cdputnam/protcalc.html>). Sequence alignment was performed using Multalin <sup>18</sup> (<http://npsa-pbil.ibcp.fr/>) and ClustalX software <sup>69</sup>. Phylogenetic tree was constructed using the TREEVIEW software <sup>59</sup>.

**Protein purification and gel filtration.** The *sav* gene of phage p2 (nucleotides 1005 to 1388 in the GenBank sequence acc.nr. FJ010786) was cloned into the His-tag vector pQE-70 (Qiagen) to create plasmid pJH12 in *E. coli* JH-65. The cloning was performed according to the recommendations in The QIAexpressionist manual using the promoter and ribosome binding site (RBS) of the vector. All cloned DNA fragments were verified by sequencing. The C-terminally His-tagged SaV was purified and subsequently run on a gel-filtration column using the same conditions recently described <sup>33</sup>.

**TABLE 1.** List of bacteria, phages and plasmids used in the study

Bacterial strain, phage or plasmid	Relevant characteristics <sup>a</sup>	Source
<b><i>L. lactis</i></b>		
IL1403	<i>L. lactis</i> subsp. <i>lactis</i> IL1403, Host for phages P008, bIL170 and bIL67	7
MB112	<i>L. lactis</i> subsp. <i>cremoris</i> MG1363, $\Delta_{upp}$ , host for phages p2, sk1, jj50, and c2	50
NZ9000	<i>L. lactis</i> subsp. <i>cremoris</i> MG1363, <i>pepN::nisRK</i>	40
JH-20	MB112 (pJH2); Cam <sup>R</sup> , Abi <sup>+</sup>	35
JH-22	IL1403 (pJH2); Cam <sup>R</sup> , Abi <sup>+</sup>	35
JH-32	MB112 (pGhost9::ISSI insert upstream of <i>abiV</i> on chromosome); Erm <sup>R</sup> , Abi <sup>+</sup>	35
JH-74	NZ9000 (pJH14); Cam <sup>R</sup>	This study
JH-76	NZ9000 (pNZ8010); Cam <sup>R</sup>	This study
JH-86	MB112 (pJH13, pAJ80); Cam <sup>R</sup> , Tet <sup>R</sup>	This study
JH-87	MB112 (pMAP84, pAJ80); Cam <sup>R</sup> , Tet <sup>R</sup>	This study
<b><i>E. coli</i></b>		
MC1000	Laboratory strain, cloning host	64
DH5 $\alpha$	Laboratory strain, cloning host	69
AJ177	DH5 $\alpha$ (pAJ80); Cam <sup>R</sup>	DTU strain collection
M15	(pREP4), Km <sup>R</sup> , used for cloning of pQE-70 His-tag vector	Qiagen
JH-60	AJ177 (pJH13); Cam <sup>R</sup> , Tet <sup>R</sup>	This study
JH-65	M15 (pJH12); Amp <sup>R</sup> , Km <sup>R</sup>	This study
JH-77	M15 (pQE-70); Amp <sup>R</sup> , Km <sup>R</sup>	This study
JH-84	DH5 $\alpha$ (pJH15); Tet <sup>R</sup>	This study
JH-88	AJ177 (pJH15); Tet <sup>R</sup>	This study
JH-89	MC1000 (pJH14); Cam <sup>R</sup>	This study
<b>Phages</b>		
p2	Small isometric headed, 936 species	56
sk1	Small isometric headed, 936 species	14
jj50	Small isometric headed, 936 species	48
P008	Small isometric headed, 936 species	48
bIL170	Small isometric headed, 936 species	20
c2	Prolate headed, c2 species	45
bIL67	Prolate headed, c2 species	72
p2.1	Small isometric headed, 936 species, deletion in orf26	This study
sk1.1	Small isometric headed, 936 species, nonsense mutation in orf26	This study
jj50.1	Small isometric headed, 936 species, nonsense mutation in orf25 (homologue to p2 orf26)	This study
P008.1	Small isometric headed, 936 species, nonsense mutation in orf33 (homologue to p2 orf26)	This study
bIL170.1	Small isometric headed, 936 species, nonsense mutation in e24 (homologue to p2 orf26)	This study
c2.1	Prolate headed, c2 species, mutation in e11 (homologue to p2 orf26)	This study
bIL67.1	Prolate headed, c2 species, mutation in orf8 (homologue to p2 orf26)	This study

**TABLE 1.** List of bacteria, phages and plasmids used in the study

Bacterial strain, phage or plasmid	Relevant characteristics <sup>a</sup>	Source
<b>Plasmids</b>		
pAB223	vector containing genetic switch of phage TP901-1	12
pAJ80	CI repressor from phage TP901-1 cloned in pCI372	46
pJH2	<i>abiv</i> cloned in shuttle expression vector pLC5; Cam <sup>R</sup>	35
pJH12	1005 to 1388 fragment cloned in pQE-70; Amp <sup>R</sup>	This study
pJH13	872 to 1446 fragment cloned in pMAP84; Tet <sup>R</sup>	This study
pJH14	872 to 1446 fragment cloned in pNZ8010; Cam <sup>R</sup>	This study
pJH15	corresponding to 872 to 1446 fragment but from p2.1 cloned in pNZ8010; Cam <sup>R</sup>	This study
pMAP84	P <sub>L</sub> promoter of phage TP901-1 cloned in pTPL (Fig. 1); Tet <sup>R</sup>	This study
pNZ8010	<i>PnisAgusA cat</i> ; Cam <sup>R</sup>	23
pTPL	low-copy <i>E. coli</i> – <i>L. lactis</i> shuttle and promoter cloning vector; Tet <sup>R</sup>	62
pQE-70	Cloning vector for C-terminal His-tagging of proteins; Amp <sup>R</sup>	Qiagen
<sup>a</sup> Fragment refers to GenBank accession number FJ010786; Abi <sup>+</sup> , phage resistance phenotype; Amp <sup>R</sup> , ampicillin resistance; Cam <sup>R</sup> , chloramphenicol resistance; Erm <sup>R</sup> , Erythromycin resistance; Km <sup>R</sup> , Kanamycin resistance, Tet <sup>R</sup> , Tetracycline resistance.		

**Cloning strategies.** To express *sav* in *trans* in *L. lactis*, we first tried the synthetic promoter strategy <sup>67</sup>. A forward primer was used

(5'-CCGCTCGAGNNNNNTATTCTTNACANNNNNNNNNNNNNNNNNNNTATNATNNNNNC  
ATTACATGGATTGGGGGTAACATCTA**ATGA**ATTATGGTACAAATAA-3'),

which encompasses a randomized promoter region of p2 *orf30* (GenBank FJ010786) fused to a part of *sav* (underlined) including the start-codon (bold face) and the ribosome-binding site. Using a reverse primer, a DNA fragment corresponding to nucleotides 980 to 1403 (GenBank acc.nr. FJ010786) was amplified by PCR and attempted cloned into the promoter-less shuttle-vector pTPL <sup>55</sup>. The cloning of a 1.4-kb DNA fragment from phage p2 containing the five genes (*orf30*, *orf29*, *orf28*, *orf27*, and *sav*) into pTPL was also attempted. Both cloning experiments were unsuccessful.

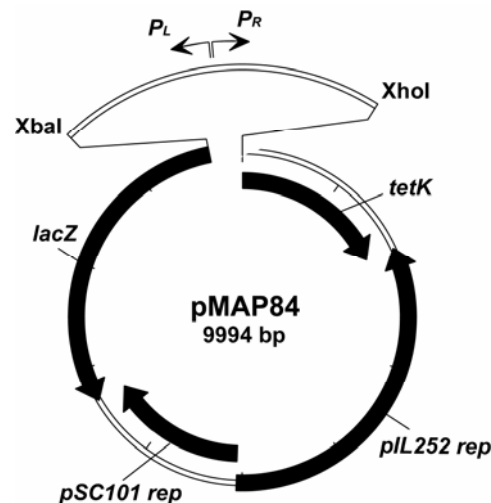
A 0.6-kb fragment from phage p2, corresponding to *sav* and the upstream gene *orf27* (nucleotide 872 to 1446 in Genbank FJ010786), was amplified by PCR and successfully cloned into the newly constructed expression vector pMAP84 (pJH13) using the *E. coli* strain AJ177, in which expression is repressed (see below). The same 0.6-kb fragment was also successfully cloned in the nisin inducible vector pNZ8010 <sup>21</sup> (pJH14). Finally, the mutated *orf27-sav* region of phage mutant p2.1 was also PCR amplified and successfully cloned into pMAP84 (pJH15).

**Construction of the expression vector, pMAP84.** The low-copy promoter-less shuttle-vector pTPL <sup>55</sup> was used to construct the novel expression vector pMAP84. A DNA fragment encompassing nucleotides 3142 to 3458 in the lactococcal phage TP901-1 (GenBank acc.nr.

[NC\\_002747](#)) was amplified by PCR using vector pAB223 as template <sup>11</sup>. This DNA fragment containing the  $P_L$  promoter region from the genetic switch in TP901-1 <sup>42</sup> was inserted in the multiple cloning site of pPTPL using the restriction sites XhoI and XbaI so that the  $P_L$  promoter points towards the LacZ reporter gene in the vector (Fig. 1).

**Induction experiments.** *L. lactis* cultures containing either pJH14 or pNZ8010 were grown exponentially to  $OD_{600} = 0.1$  at which point each culture was split in two sub-cultures. At T=0 nisin ( $0.25 \text{ ng ml}^{-1}$ ) was added to one of the sub-cultures and samples for  $OD_{600}$  and surviving cells (CFU/ml) were taken after 5, 35 and 135 minutes (Fig. 5A+B). A similar experiment was performed with *E. coli* JH-65, which contained *sav* cloned into the IPTG inducible vector pQE-70 and *E. coli* JH-55 as a control, which had only pQE-70 vector. At  $OD_{600} = 0.2$  (T = -2), IPTG was added ( $0.5\text{mM}$ ) and samples were taken after 2 minutes (T=0) as well as after 30, 90, and 180 minutes for  $OD_{600}$  measurements and cell counts (Fig. 5C+D).

**Nucleotide accession numbers.** A sequence was submitted to the GenBank database under the accession number FJ010786.



**Fig. 1.** Construction of the pMAP84 vector by cloning the promoter region of the TP901-1 genetic switch in the multiple cloning site of pPTPL

## RESULTS

**Isolation of phage mutants insensitive to AbiV.** Phage p2 is a small-isometric-headed phage of the 936-like group that propagates on *L. lactis* subsp. *cremoris* MG1363. This phage was recently shown to be sensitive to AbiV<sup>33</sup>. When propagated on AbiV<sup>+</sup> *L. lactis* cells (JH-20), phage p2 forms small plaques with an efficiency of plaquing (EOP) of 10<sup>-4</sup>. One of these small plaques was picked up at random and chosen for further characterization. The phage mutant (named p2.1) was purified three times on *L. lactis* JH-20 and its insensitivity to AbiV was confirmed by EOP assays. The above isolation procedure was similarly applied to obtain insensitive mutants of the following AbiV-sensitive wild-type phages: sk1 and jj50 (936 species, host *L. lactis* MG1363), P008 and bIL170 (936, host *L. lactis* IL1403), c2 (host *L. lactis* MG1363) and bIL67 (c2 species, host *L. lactis* IL1403). The AbiV-insensitive phage mutants were named: sk1.1, jj50.1, P008.1, bIL170.1, c2.1 and bIL67.1, respectively.

**Microbiological analyses of phage mutant p2.1.** This phage mutant was characterized for EOP, ECOI, burst size, latency, and plaque size on AbiV<sup>+</sup> and AbiV<sup>-</sup> cells. Values for EOP, ECOI, burst size, and latency time were similar with both hosts (Table 2). Moreover, they were also similar to those obtained with the wild-type phage p2 on the sensitive host *L. lactis* MB112<sup>33</sup>. Nonetheless, the plaque size of phage p2.1 was slightly smaller (0.7-1 mm) on AbiV<sup>+</sup> cells as compared to AbiV<sup>-</sup> cells (1 mm). By comparison, the plaque size of the wild-type phage p2 on AbiV<sup>-</sup> cells is 1.5 mm. These data indicated that the mutation in phage mutant p2.1 conferred a complete in-sensitive phenotype to AbiV and that the fitness cost was small.

**TABLE 2.** Effect of AbiV on lactococcal phage p2.1

Assay	<i>L. lactis</i> MB112 (AbiV <sup>-</sup> )	<i>L. lactis</i> JH-20 (AbiV <sup>+</sup> )
EOP <sup>a</sup>	1.0	1.1
ECOI (%) <sup>b</sup>	100.0	94.0 ± 25.3
Burst size (pfu/cell) <sup>c</sup>	38.8 ± 5.7	41.1 ± 4.6
Latency time (min) <sup>c</sup>	20-30	20-30
Plaque size (mm)	1	0.7 - 1

<sup>a</sup>EOP of phages p2 is 1.0 on *L. lactis* subsp. *cremoris* MG1363 (MB112).  
<sup>b</sup>MOI = 0.5, n=3, ECOI of phages p2 is 1.0 on *L. lactis* subsp. *cremoris* MG1363 (MB112).  
<sup>c</sup>MOI = 0.2, n=3



**An early transcribed gene is involved in the sensitivity to AbiV.** Genomic DNA was isolated from the phage mutant p2.1, digested with EcoRV and the restriction profile compared to a restriction map of the wild-type phage p2. No difference was observed, indicating that no large deletion or genomic re-arrangement was responsible for the AbiV insensitive phenotype (data not shown). The entire genome was then sequenced and compared to the wild-type phage p2. A comparative genome analyses revealed a total of three point mutations as well as a deletion of 56 nucleotides in the p2.1 genome (Fig. 2). All the point mutations and the deletion were located within a 255 bp region of the genome, which was previously shown to be early transcribed<sup>12,13</sup>. Two point mutations were located in the *orf26* leading to amino acid changes (A45T and A60D), and one point mutation was located upstream of *orf27* (Fig. 2). The deletion covered the 3'-end of *orf27*, as well as the ribosome binding site (RBS) and start codon of *orf26*. The deletion occurred between two GGTA sequences and led to a translational in-frame fusion of *orf27* and the downstream *orf26* in the phage mutant p2.1 (see below).

A BLAST analysis of *orf26* revealed similarity to the following lactococcal phage genes: *orf25* in jj50 (GenBank acc.nr. [NC\\_008371](#)), *orf26* in sk1 (GenBank acc.nr. [NC\\_001835](#)), *orf33* in P008 (GenBank acc.nr. [NC\\_008363](#)), *e24* in bIL170 (GenBank acc.nr. [NC\\_001909](#)), as well as *e11* in phage c2 (GenBank acc.nr. [NC\\_001706](#)), and *orf8* in bIL67 (GenBank acc.nr. [L33769](#)). All these phages containing the *orf26* homologue are sensitive to AbiV<sup>33</sup>. Interestingly, an *orf26* homologue is absent in the genome of the phage 712 (936-species) which is insensitive to AbiV. Similarly, lactococcal phages of the P335 species, which are also insensitive to AbiV, do not contain an *orf26* homologue<sup>33</sup>.

Since mutations were only found in *orf27* and *orf26* of p2.1 genome, this region was sequenced in the other isolated phage mutants (jj50.1, sk1.1, P008.1, bIL170.1, c2.1, bIL67.1) and compared to the wild-type sequences. Comparative analyses revealed only point mutations in the *orf26* homologue of all the phage mutants (Table 3) and no mutations in the *orf27* homologue (data not shown). It was therefore concluded that *orf26* of phage p2 is involved in sensitivity to AbiV and the gene was renamed *sav* (mnemonic for sensitivity to AbiV).

		10	20	30	40	50	60
		G I G F T L I A I T W I G I I A T L L I					
p2		TAGGTATAGGCTTTACACTTATCGCTATCACTTGGATAGGTATAATTGCAACGTTGCTTA					
p2.1		TAGGTATAGACTTTACACTTATCGCTATCACTTGGATAGGTA-----					
		*				*****	
		70	80	90	100	110	120
				orf27 stop and sav start			
		T W I G G N I					
p2		TTACATGGATTGGGGGTAACATCTAATGAATTATGGGTACAAATAAGCACTATGCCAATGA					
p2.1		-----CAAATAAGCACTATGCCAATGA					
		M N Y G T N N H Y A N E					
		*****					
		130	140	150	160	170	180
p2		ATACGGTATGGAACCTTAACGAATACTTTAAACATCATTTTAGCTATGAAGAGCTTGCAGG					
p2.1		ATACGGTATGGAACCTTAACGAATACTTTAAACATCATTTTAGCTATGAAGAGCTTGCAGG					
		Y G M E L N E Y F K H H F N Y E E L A G					
		190	200	210	220	230	240
p2		CTGGTATACAATGCAGGTATTAAAGTATCTAGTGAGAGCTGGCAAGAAAGAGGGTGAAAG					
p2.1		CTGGTATACAATGCAGGTATTAAAGTATCTAGTGAGAACTGGCAAGAAAGAGGGTGAAAG					
		W Y T M Q V L K Y L V R A G K K E G E S					
					*		
		250	260	270	280	290	300
p2		CTACGACAAAGACCGTAACAAGGCTTTAGACTATGCAGGAGAACTTGCTAACTTAAGTAA					
p2.1		CTACGACAAAGACCGTAACAAGGATTTAGACTATGCAGGAGAACTTGCTAACTTAAGTAA					
		Y D K D R N K A L D Y A G E L A N L S N					
				*			
		310	320	330	340	350	360
p2		CGAGAATGAGCTTACAGAATACACTACTGACGACATTATGGGCTTTGCACAAGATATAGC					
p2.1		CGAGAATGAGCTTACAGAATACACTACTGACGACATTATGGGCTTTGCACAAGATATAGC					
		E N E L T E Y T T D D I M G F A Q D I A					
		370	380	390	400	410	420
p2		TGATGATTTCAAACAATGGAAAGGCGAAAGAAATAACTTTAAATCAGAGTTCACGAAAGA					
p2.1		TGATGATTTCAAACAATGGAAAGGCGAAAGAAATAACTTTAAATCAGAGTTCACGAAAGA					
		D D F K Q W K D E R N N F K S E F T K E					
		430	440	450	460	470	
p2		AGAGATAAAAGCGATTGATGAAAGATACTTGGAAATTTATTGAAGAGGTCTAA					
p2.1		AGAGATAAAAGCGATTGATGAAAGATACTTGGAAATTTATTGAAGAGGTCTAA					
		E I K A I D E R Y L E F I E E V					

**Fig. 2.** Localization of mutations in the genome of phage mutant p2.1. Mutations are marked with an asterisk and include three point mutations and a 55 nt deletion which encompasses the ribosome binding site (RBS) and *sav* start codon. RBS and start codon are underlined in wild type sequence and *orf27* stop as well as *sav* start codons are in bold face. Deduced SaV amino acid sequence is written below nucleotide sequence while the deduced sequence for ORF27 is written above.

**Mutations in SaV.** The point mutations in the *sav* homologues of all the phage mutants were analyzed for effects on the translated SaV (Table 3). Following changes in the amino acid sequence were observed: G-65-stop (jj50.1), M-1-I (sk1.1), W-33-stop (P008.1), E-58-stop (bIL170.1), T-48-P (c2.1), and Y-10-stop (bIL67.1). Most phage mutants had a nonsense mutation in *sav* and two mutants (p2.1 and sk1.1) had a deleted start codon. The mutation in phage c2.1 caused a single amino acid change (T-48-P), most likely leading to a conformational change in the protein. Interestingly, this point mutation is located in a region of the protein, which has a high degree of conservation despite the otherwise large differences between the homologues of the c2- and 936-like phages (Fig. 3). Moreover, the majority of the mutations in the different phage mutants are located in this region indicating that it serves an important function for the sensitivity to AbiV. Taken altogether, the nature of the point mutations suggests that the gene product SaV is involved in AbiV sensitivity.

**TABLE 3.** *sav* homologues in phage mutants insensitive to AbiV

Phage	<i>orf</i>	Amino acids	Mutation (nt)	Effect (aa)
p2.1	26	128	---	55nt deletion
			GCT→ACT	A-45-T
			GCT→GAT	A-60-D
sk1.1	26	128	ATG→ATA	M-1-I
jj50.1	25	128	GGA→TGA	G-65-stop
P008.1	33	119	TGG→TAG	W-33-stop
bIL170.1	e24	130	GAA→TAA	E-58-stop
c2.1	e11	122	ACT→CCT	T-48-P
bIL67.1	8	116	TAC→TAA	Y-10-stop

**Analyses of the *sav* gene.** The *sav* gene in wild-type phage p2 consists of 384 bp and its gene product contains 128 amino acids (15.3 kDa). The *sav* gene marks the end of a putative 5-gene operon consisting of *orf30* to *orf26* (*sav*) (GenBank acc.nr. FJ010786). The region shares 97 % nucleotide identity with a region (nucleotides 17543 to 18862) found in the genome of lactococcal phage sk1 (GenBank acc.nr. [NC\\_001835](#)) for which DNA sequence<sup>13</sup> and transcription analyses<sup>12</sup> are available. Preceding *orf30* is a lactococcal consensus promoter and there are overlaps between *orf28*, *orf27*, and *sav*. Indeed, the start codon of *sav* overlaps with the stop codon of the upstream gene *orf27* (Fig. 2) and the start/stop codons of *orf27/orf28* also overlap, which suggests translational coupling of the gene products. The start codon of all the other *sav* homologues was overlapping the stop codon of a small upstream gene except for the *orf26* homologue in phage c2,

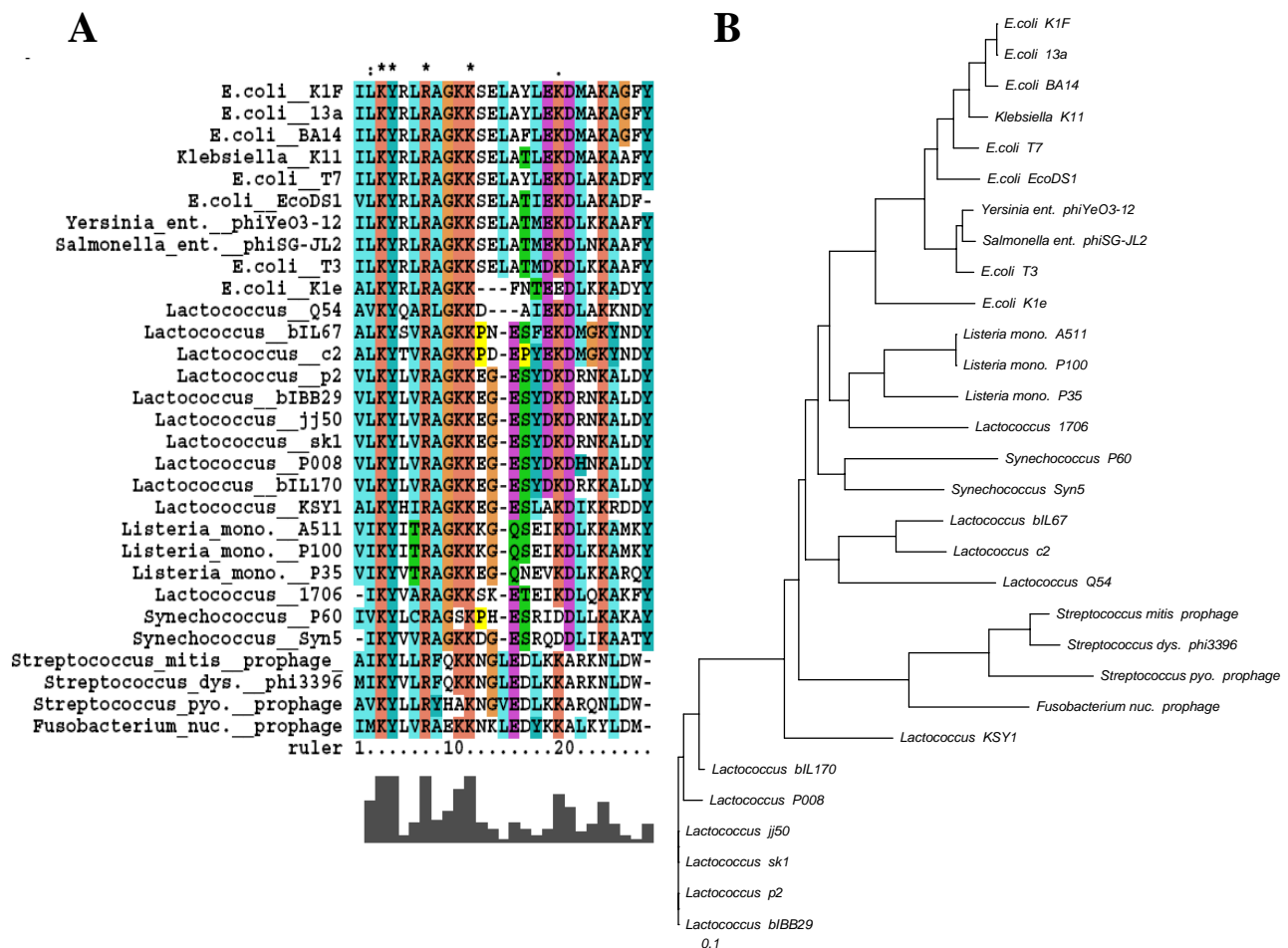
which was separated (6 bp) from the upstream gene. The start codon of *sav* was preceded by a putative ribosome-binding site (GGATTGGGGGT, underlined sequence matches *Lactococcus* consensus). However, the RBS of *orf27* (ACTTAGGAGGA) and *orf28* (ACTAAGGAGAA), appeared closer to the consensus sequence and may therefore be stronger than the RBS of *sav*, supporting a possible translational coupling. This was further supported by estimated secondary structures of mRNA covering RBS of the three genes. These structures had calculated dG values of -6 kcal/mol, -9 kcal/mol, and -19 kcal/mol for *orf28*, *orf27*, and *sav*, respectively indicating that *sav* needs resolution of upstream RNA by ribosomes terminating translation of upstream *orf27*.

In lactococcal phage mutant p2.1, a 56-nucleotide deletion (Fig. 2) covered the 3'-end of *orf27* (117 bp, 39 aa, 4.2 kDa) as well as the RBS and the 5'-end of *sav*. This deletion created an in-frame fusion between *orf27* and *sav*, generating a fused protein of 17.6 kDa.

	10	20	30	40	50	60
p2	-----	MNYGTNKH	YANEYGM	ELNEYFKH	HFSYEEL	AGWYTMQ
p2.1	-----	<b><u>MNYGT</u></b> NKH	YANEYGM	ELNEYFKH	HFSYEEL	AGWYTMQ
jj50.1	-----	MNYGTNKH	YANEYGM	ELNEYFKH	HFSYEEL	AGWYTMQ
sk1.1	-----	<b><u>IN</u></b> YGTNNH	YANEYGM	ELNEYFKH	HFN	YEELAGW
P008.1	-----	MNFKKNRH	YANEYGV	ELNEYFKH	NFN	YEELAGX
bIL170.1	MAWRQY	IMNFKEN	KHYANKY	GV	ELNEYLK	HNFN
c2.1	MAQDY	YANKYGI	QLEEF	LIWGSE	WDLKF	-WKYNFT
bIL67.1	MAKD	YYANKX	GIQLE	EFLVWG	SEWDLKF	-WQYNFT
	70	80	90	100	110	120
p2	LDYAGELANLS	SNENELTEY	TTDDIMGFA	QDIADDFKQ	WKGERNNFK	SEFTKEEIK
p2.1	LDYAGELANLS	SNENELTEY	TTDDIMGFA	QDIADDFKQ	WKGERNNFK	SEFTKEEIK
jj50.1	LDYAGELANLS	SNENELTEY	TADDIMGFA	QDIADDFKQ	WKGERNNFK	SEFTKEEIK
sk1.1	LDYAGELANLS	SNENELTEY	TTDDIMGFA	QDIADDFKQ	WKDERNNFK	SEFTKEEIK
P008.1	LDYAKELAKLS	SNENELTEY	TTEDIMGFT	QDMADDFKN	WKGE-----	
bIL170.1	LDYAGELANLS	NDNELTEY	TTDDIMVFI	QELADDFER	WEGIK-----	
c2.1	NDYIDMAVKMG	FE----R	SEAEDWVAL	QKSI---	FEEFKGKKA	EIEELEKR
bIL67.1	NDYINMAVKMG	FE----Q	VEAENWVAL	QKSI---	FEEFKGKKA	ELEEIRKRE

Fig. 3. Alignment of amino acid sequences of SaV from phage mutants insensitive to AbiV. Mutations are underlined and in bold face: line-through means deletion, X means stop-codon.

**Analyses and overexpression of the SaV protein.** A His-tagged SaV protein (15.3 kDa) of phage p2 was produced by IPTG induction of exponentially growing *E. coli* cells and subsequently harvested and purified. The molecular weight of the purified native SaV protein was estimated at  $33 \pm 0.7$  kDa by gel-filtration (data not shown), suggesting that SaV forms a dimer in its native form. The protein consists of ca 84 %  $\alpha$ -helixes and 16 % coils but no putative DNA-binding helix-turn-helix motifs were identified by bioinformatics analyses. No similarity to proteins of known functions as well as no conserved domains was found in SaV. However PSI-BLAST (5 iterations) revealed that the conserved region observed in the SaV homologues in 936-like and c2-like phages (Fig. 3) is also present in proteins from phages infecting other hosts such as *Klebsiella pneumoniae*, *E. coli*, *Yersinia enterocolitica*, *Salmonella enterica*, *Listeria monocytogenes*, *Fusobacterium nucleatum*, *Streptococcus* sp., and *Synechococcus* sp. (Fig. 4).



**Fig. 4.** Alignment of conserved region in SaV homologues of different phages using ClustalX. Bar diagram presents degree of conservation (A). Bootstrapped phylogenetic tree of the conserved region in SaV (B).

**SaV is toxic to *L. lactis* and *E. coli* cells.** It was investigated whether AbiV efficiency could be decreased or increased by expressing *sav* in *trans*. We first tried the strategy of a synthetic promoter library (SPL) to modulate the expression of *sav*<sup>67</sup>. A DNA fragment including only *sav* behind a randomized promoter region was successfully obtained and cloning was attempted in both *L. lactis* MB112 and *E. coli* DH5 $\alpha$  using the low-copy promoter-less shuttle vector pPTPL<sup>55</sup>. No transformants were obtained suggesting that *sav* might be toxic when expressed in these two bacteria. Cloning of a region encompassing *orf30* to *sav*, which includes a lactococcal consensus promoter upstream of *orf30*, into pPTPL was also unsuccessful in *E. coli* MC1000.

To confirm the possible toxicity of *sav*, we first constructed a new vector with regulated gene expression, which was named pMAP84 (Fig. 1). In this vector, transcription from the P<sub>L</sub> promoter can be repressed by the lactococcal phage TP901-1 repressor protein CI expressed from a separate vector<sup>42</sup>. A PCR fragment encompassing *sav*, *orf27* and the upstream region with RBS was ligated in pMAP84 (to form pJH13) and the resulting plasmid was transformed in *E. coli* DH5 $\alpha$  and *E. coli* AJ177 (expressing the CI repressor). No transformants were obtained in DH5 $\alpha$  whereas cloning was successful in AJ177 (JH-60). To test if pJH13 could exist within the cell without repression by CI (pAJ80), total plasmid (pJH13 and pAJ80) was extracted from *E. coli* JH-60 and used to transform *E. coli* MC1000 and *L. lactis* MB112, followed by tetracycline selection (only selection for pJH13). Fifty *E. coli* and 100 *L. lactis* tetracycline-resistant transformants were tested for tetracycline and chloramphenicol resistance. All transformants were resistant to both antibiotics indicating that co-transformation of pJH13 and pAJ80 had occurred in all cases. We therefore concluded that pJH13 alone is toxic to the cells.

A cell growth experiment was conducted using lactococcal cells expressing the CI repressor and either pJH13 or the cloning vector alone pMAP84. The cells containing both plasmids had a 50 % reduced growth rate and a 15 % reduced final yield as compared to cells with only the empty vector. The same experiment was conducted with *E. coli* cells harbouring a repressed *sav* (JH-60) or expressing the mutated *sav* (JH-84). Similarly, cells of *E. coli* JH-60 (SaV<sup>+</sup>) had a 50 % reduced growth rate and a 15 % reduced final yield as compared to cells of *E. coli* JH-84 (data not shown). The above results strongly indicate that SaV is toxic in both *E. coli* and *L. lactis*, even when expressed at low levels.

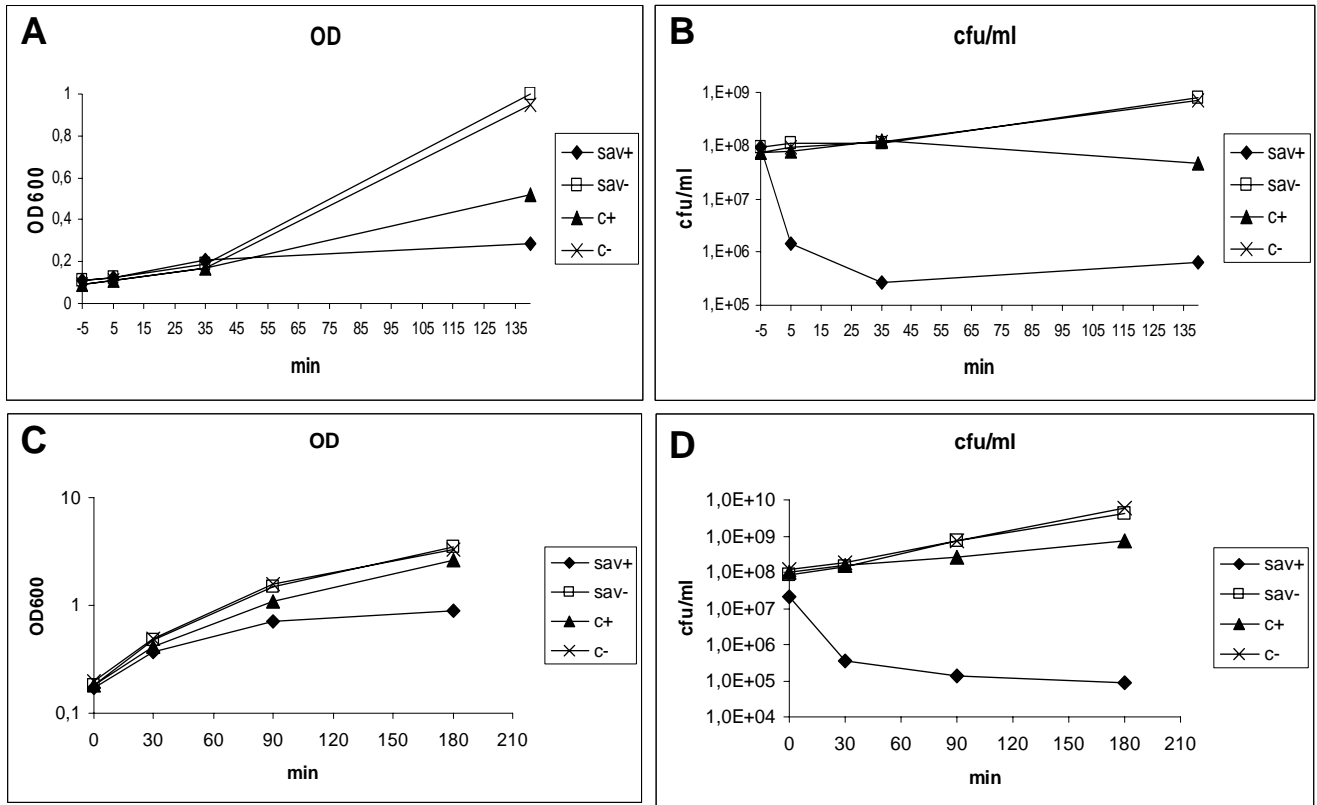
Further, we wanted to determine if the co-existence of AbiV and repressed SaV in the same cell was possible. The plasmids pJH13 and pAJ80 were transformed in cells expressing Abi. The transformation was done in hosts with low (*L. lactis* JH-32) and high (*L. lactis* JH-20) levels of

AbiV expression<sup>33</sup>. PCR analysis of 40 colonies from each transformation showed an intact *sav* but a deleted *abiV* in all transformants. These observations were supported by sequencing and phage resistance tests, which showed that all the mutants had become sensitive to phage p2 (data not shown).

Finally, a cloning experiment was made where a DNA fragment encompassing *orf27-sav* was PCR-amplified from phage mutant p2.1 using the same primerset as for the wild type cloning. This fragment was successfully cloned in the vector pMAP84 in the *E. coli* strains AJ177 (pAJ with CI repressor) and MC1000 (no repressor), yielding strains JH-88 and JH-84, respectively. The successful cloning of the unrepressed *orf27-sav* region from phage p2.1 (JH-84) therefore suggests that the toxicity of *sav* towards *E. coli* is due to the mutations that also renders the phage insensitive to AbiV.

**Induction of *sav* causes rapid cell death.** A nisin inducible expression system was used to time the expression of *sav* in *L. lactis*. A PCR amplified fragment containing *orf27-sav* and RBS was ligated in pNZ8010<sup>21</sup> (pJH14) and cloned in *E. coli* MC1000 (JH-89). The construct was then transferred to *L. lactis* NZ9000 (JH-74) used for the nisin induction experiments<sup>20</sup>. Due to the toxic properties of nisin, the nisin-induced control (Fig. 5A+B, c+) reached only 50 % of the cell density obtained in the non-induced cultures (Fig. 5A+B, *sav*- and c-). In the induced *sav* cultures, the OD<sub>600</sub> rose from 0.1 to 0.2 in the first 30 minutes after which no further increase of OD<sub>600</sub> was observed and a number of cells rapidly died. Five minutes after the induction, the number of cells able to form colonies on normal GM17 plates was reduced 100-fold. It was further reduced 10-fold after 35 minutes and ended at 0.5 % of the original cell count after 135 minutes.

A similar experiment was performed with the IPTG inducible *E. coli* clone JH-65 overexpressing C-terminal His-tag SaV (see above). The IPTG induced control (Fig. 5 C+D, c+) was only marginally inhibited during the experiment. However, the number of surviving SaV<sup>+</sup> cells was severely reduced following the induction (Fig. 5D). After 30 minutes, the cell count was reduced 500-fold and ended at a 1000-fold reduction after 180 minutes. The above results strongly indicate that the expression of *sav* has a strong and fast-functioning anti-microbial effect.



**Fig. 5.** Growth (A/C) and surviving cells (B/D) after induction of *sav* with nisin or IPTG in *L. lactis* (A/B) and *E. coli* (C/D), respectively. Sav indicates vector pNZ8010 with cloned *sav* and c indicates empty vector, + and – indicate induction and no induction with nisin at T0 or IPTG at T2, respectively



## DISCUSSION

Lactococcal phage mutants that are insensitive to the abortive infection mechanism AbiV can be obtained at a frequency of  $10^{-4}$ . The analysis of these phage mutants was used in the present study to further investigate the mode of action of AbiV. The phage mutant p2.1 was randomly chosen for whole genome sequencing. Mutations and a deletion were found in a 255 nt region as the only difference with the wild-type genome. One gene was particularly affected by these genetic changes and was named *sav*. This is presumably the last of an early-transcribed five-gene cluster (GenBank acc.nr. FJ010786). This genomic region has 97 % nucleotide identity with a confirmed 5-gene operon (*orf30* to *orf26*) found in phage sk1<sup>12</sup>. This cluster is transcribed from a consensus promoter<sup>72</sup> upstream of *orf30*<sup>13</sup> which is likely recognized by host RNA polymerase and typically found in the early transcribed region of lactococcal phages<sup>12</sup>.

Sequence data from the *sav* region in other AbiV insensitive lactococcal phage mutants from both 936 and c2 species revealed a high frequency of nonsense mutations as well as significant amino acid changes in a region of the protein, which is highly conserved among SaV homologues of 936-like and c2-like phages (Fig. 3). The point mutation in phage c2.1 (T-48-P) most likely induces a conformational change in the conserved region of the polypeptide. Furthermore, two point mutations in phage p2.1 led to radical amino acid changes (A-45-T and A-60-D), which might also alter the property of the polypeptide due to the changes from neutral alanine (A) to polar (T and D) and charged (D) residues. Although the phage mutant p2.1 also contains a 56 nt deletion which prevents expression of native SaV, it creates an in-frame translational fusion of N-terminal ORF27 (codon 1 to 25) with an almost complete SaV (residue 4 to 128). Such rearrangement appears to have an effect, albeit limited, on the phage lytic cycle and may not have affected the function of SaV. On the other hand, some of the mutations likely led to the production of a significantly altered SaV protein, which rendered the phage insensitive to AbiV while still allowing the assembly of functional phages.

Analysis of the secondary structure of SaV revealed a high (84 % and 74 %) content of  $\alpha$ -helixes in phages p2 and c2, respectively. The distribution of  $\alpha$ -helixes (interspersed with random coil) was remarkably similar in the two phages despite the lack of sequence similarity (Fig. 3). Furthermore, a coil in the middle of the conserved region is significantly prolonged by the two amino acid changes in phage p2.1. It is tempting to speculate that this conserved region might be a target site for the activity of AbiV. Interestingly, when PSI-BLAST was used to search for more

distant SaV homologues, similarity was found to putative proteins from phages infecting a very diverse range of bacterial hosts (gram-positive, gram-negative, and cyanobacteria). The similarity was primarily observed in the conserved region and an alignment of this region revealed a high degree of conservation in specific residues (Fig. 4A). A phylogenetic analysis suggested that this SaV-like region has evolved from a common ancestor. Nonetheless at this time, no putative function could be assigned to SaV though the conservation of the middle region of SaV suggests a common function in a wide range of phages.

The start-codon of *sav* overlaps with the stop-codon of the upstream gene *orf27* (Fig. 2). Such out-of-phase overlapping genes are often observed in phages, which are known to have compact genomes<sup>52,63</sup>. Terminal overlap could cause translational coupling<sup>57</sup>, a mechanism that ensures the tightly coupled translation of the two overlapping genes<sup>63</sup>. Terminal overlap of *sav* with an upstream gene of about half the size was observed in all the investigated AbiV sensitive lactococcal phages, except for phage c2 where the two genes were separated by 6 bp. Similarly, 77 % of the homologous *sav* genes reported in Fig. 4 overlap with an upstream gene. It is therefore tempting to speculate that *orf27* and *sav* are translationally coupled. This ensures an equimolar ratio of the two polypeptides and may not entail a direct functional relation of them. However, in case of a terminal overlap, it is generally observed that the two genes almost always encode structurally or functionally related polypeptides<sup>63</sup>. Translational coupling has been demonstrated for phage structural genes<sup>15,31,64</sup> but also for polypeptides in the DNA polymerase holoenzymes of coliphage T4<sup>70</sup> and lactococcal phage sk1<sup>13</sup>.

To study the interaction of SaV with AbiV, we tried to complement SaV from the wild-type phage p2 by providing the gene in *trans* during infection with the AbiV-insensitive phage mutant p2.1<sup>4,24</sup>. The *sav* cloning experiments together with subsequent SaV induction experiments showed that the expression of the protein causes cell death (Fig. 5). It has been demonstrated in a number of cases that gene products from early-transcribed phage genes can have antimicrobial activity when expressed in bacterial cells<sup>51,44,55,56,58</sup>. These phage proteins produced early after the beginning of the infection have evolved to quickly inactivate or redirect critical processes of the bacterial cell machinery in order to shut down host metabolic activities and facilitate production of phage components<sup>37,38,41,50</sup>. For example, early phage proteins have been demonstrated to inhibit transcription in *E. coli* by interaction with RNA polymerase<sup>51</sup>,  $\sigma$ 70 transcription factor<sup>58</sup> or to redirect DnaB helicase to favour phage DNA replication<sup>44,55,56</sup>. The very quick toxic effects in both *L. lactis* and *E. coli* indicate that SaV might be such an early regulatory protein that targets a critical

cellular mechanism common to the two distantly related bacterial species. At this time, the molecular mode of action of SaV is unknown. However, the expression of the mutated ORF27-SaV from phage p2.1 was not toxic in *E. coli*, which indicates that the mutations in the conserved region of SaV rendering the phage insensitive to AbiV is also involved in the antimicrobial activity of the protein. It is therefore likely that it is the translated SaV polypeptide, which is somehow involved in both the AbiV phenotype and toxicity towards the host cell.

In conclusion, the early transcribed phage protein SaV was demonstrated to be involved in the sensitivity of 936- and c2-like phages to the recently described abortive infection mechanism AbiV. The middle region of SaV is conserved and evolutionary related among distantly related phages. Due to its fast-working toxic effect in both *L. lactis* and *E. coli*, we suggest that the function of SaV during the phage infection is to shut down or re-direct essential host functions.

## **ACKNOWLEDGMENTS**

We thank Margit Pedersen and Cengiz Binici for constructing the pMAP84 vector. This work was funded in part by a graduate scholarship from the Technical University of Denmark to JH and a strategic grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada to SM.

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# **MANUSCRIPT III**



# Direct interaction between the lactococcal abortive infection protein AbiV and the phage protein SaV prevents translation of phage proteins

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## ABSTRACT

In this study, we characterized the interaction between the novel lactococcal phage abortive infection protein AbiV and the phage protein SaV which was recently shown to be involved in AbiV sensitivity. The two dimeric proteins interact directly by forming a complex consisting of AbiV<sub>2</sub>SaV<sub>2</sub> and we propose that the otherwise non-toxic AbiV protein is activated by SaV by this interaction to inhibit the cellular translational machinery.

The Abi mechanism severely inhibited total protein synthesis in cells infected with the 936 phage p2 at an early stage of the lytic cycle while RNA synthesis was only slightly inhibited. A more detailed picture was obtained by analyzing early, middle, and late transcription as well as synthesis of specific early and late proteins during infection of AbiV<sup>+</sup> cells with phage p2. These experiments revealed only slight inhibition of early transcription (including *sav*) while translation of the *sav* gene product (SaV) was non-detectable. We therefore concluded that the AbiV mechanism functions by inhibiting translation of phage proteins at an early stage. The early shut-down of translation probably caused the inhibition of middle and late transcripts which need activation by early phage encoded activator proteins. Phage DNA replication was observed for the isometric headed 936 phage sk1 but not for the prolate headed phage c2. We propose a hypothesis to explain the mode of action of the AbiV mechanism in both 936 phages and c2 phages.

## INTRODUCTION

Bacteriophages killing susceptible *Lactococcus lactis* strains during milk fermentation is a persisting problem for the dairy industry<sup>1,47,49</sup> that primarily suffers from phage attacks of the three lactococcal phage species 936, c2 and P335<sup>12</sup>. As a defence against phage attacks, *L. lactis* has evolved numerous natural anti-phage barriers that protect the cell against phage adsorption, against injection of phage DNA, through restriction modification (R/M) systems or through abortive infection (Abi) mechanisms<sup>23,37,47</sup>. Abi mechanisms inhibit the phage lytic cycle after DNA injection and before release of phage progeny<sup>30</sup> and the 23 Abi mechanisms isolated to date are designated AbiA<sup>33</sup> to AbiZ<sup>21</sup>. They form a heterogeneous group of anti-phage mechanisms usually encoded by a single gene though some Abi systems are encoded by two<sup>4,13,14,24,51</sup> or more<sup>61</sup> genes. All the genes encoding lactococcal Abi systems have a low G+C content and they share only limited amino acid sequence similarity<sup>11</sup>. They also vary significantly in their efficiency (measured as efficiency of plaquing – EOP) as well as efficacy against the three main lactococcal phage species<sup>56</sup>. The effect of one Abi mechanisms on the phage lytic cycle can also vary according to the species of the infecting phage<sup>7,11</sup>.

The extensive industrial use of Abi systems favour emergence of Abi-insensitive phage mutants<sup>47</sup>. From an industrial point of view there is therefore a permanent need for obtaining new anti-phage barriers either by discovering new natural phage resistance mechanisms or by engineering artificial phage resistance systems<sup>55</sup>. Different engineering approaches have been applied such as production of anti-sense phage mRNA<sup>46</sup>, phage encoded resistance (PER)<sup>32</sup>, expression of the lytic repressor in temperate phages<sup>32,44</sup>, super infection exclusion (sie)<sup>45</sup>, and phage triggered suicide mechanisms<sup>17,18</sup>. However, these artificial phage resistance mechanisms generally only have limited efficiency against a small range of phages<sup>37</sup>. An alternative approach could therefore be to engineer existing Abi mechanisms to improve their efficiency or expand their range of efficacy to include more phage species. However, understanding the mode of action of a given Abi system against different phages is a prerequisite for such engineering approaches.

For most Abi systems the overall effect on the phage lytic cycle has been revealed<sup>11,20,56</sup> and for many of them, the phage gene responsible for sensitivity to the Abi mechanism has been identified by investigating Abi-insensitive phage mutants<sup>2-6,15,16,19,29,40,48</sup>. However, a deeper understanding of the molecular interactions between the Abi mechanism and the phage components

that confer the Abi phenotype will provide valuable information for designing engineered strategies to improve Abi systems.

Recently, we characterized the novel abortive infection mechanism AbiV<sup>28</sup> and the phage protein SaV with anti-microbial properties that is involved in AbiV sensitivity<sup>29</sup>.

Here we investigate the mode of action of the AbiV anti-phage system. We demonstrate the effect of AbiV on DNA replication, transcription, and translation during the phage lytic cycle. Moreover, we demonstrate that AbiV interacts directly with SaV to arrest the phage lytic cycle. Finally, we propose a model for the mode of action for AbiV that might explain observed differences in the effect on the lytic cycle of 936-phages and c2-phages.

## MATERIALS AND METHODS

**Bacterial strains, phages, plasmids and growth conditions.** Bacterial strains, bacteriophages and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37°C in LB medium <sup>54</sup> or Turbo Broth (AthenaES) for protein expression. *L. lactis* was grown at 30°C in M17 <sup>59</sup> supplemented with 0.5 % glucose (GM17). However, in experiments with incorporation of radioactive nucleosides *L. lactis* was grown in SA medium supplemented with 0.5 % glucose <sup>35</sup> and with reduced methionine concentration (5 µg ml<sup>-1</sup>) <sup>38</sup>.

During phage infection experiments, bacterial growth and lysis of phage infected cultures were determined by cell density (OD<sub>450</sub> in GSA medium and OD<sub>600</sub> in GM17 medium) using a BioScreen C apparatus (Oy Growth Curves Ab Ltd).

In phage infection experiments, 10 mM CaCl<sub>2</sub> was added to the medium. Propagation of phages and phage lysate titration was performed as described previously <sup>22</sup>. When appropriate, antibiotics were added as follows: for *E. coli*, 100 µg ml<sup>-1</sup> of ampicillin, 34 µg ml<sup>-1</sup> of chloramphenicol and 25 µg ml<sup>-1</sup> of kanamycin; for *L. lactis*, 5 µg ml<sup>-1</sup> of chloramphenicol.

**TABLE 1.** List of bacteria and phages used in the study

Bacterial strain or phage	Relevant characteristics	Source
<b>Bacteria</b>		
JH-20	<i>L. lactis</i> subsp. <i>cremoris</i> MB112 (pJH2); Cam <sup>R</sup> , AbiV <sup>+</sup>	28,34
JH-54	<i>L. lactis</i> subsp. <i>cremoris</i> MB112 (pLC5); Cam <sup>R</sup> , AbiV <sup>-</sup>	28
JH-62	<i>E. coli</i> M15 (pJH11); AbiV His-tag. Amp <sup>R</sup> , Km <sup>R</sup>	28
JH-65	<i>E. coli</i> M15 (pJH12); SaV His-tag. Amp <sup>R</sup> , Km <sup>R</sup>	29
<b>Phages</b>		
p2	Small isometric headed, 936 species	50
sk1	Small isometric headed, 936 species	10
c2	Prolate headed, c2 species	43
Cam <sup>R</sup> , chloramphenicol resistance (5 µg ml <sup>-1</sup> ); Amp <sup>R</sup> , ampicillin resistance (100 µg ml <sup>-1</sup> ); Km <sup>R</sup> , Kanamycin resistance (25 µg ml <sup>-1</sup> ); AbiV <sup>+</sup> , AbiV phage resistance phenotype; AbiV <sup>-</sup> , AbiV phage sensitive phenotype.		

**Purification of His-tagged proteins.** AbiV and SaV were previously cloned into the pQE-70 vector (Qiagen) to create C-terminal His-tags on both proteins in *E. coli* M15 strains (strains JH-62 and JH-65, respectively). Proteins were purified using the following protocol: After over night induction at 25°C with 0.5mM IPTG cells were harvested by centrifugation for 10 min at 4000×g. The pellet was resuspended in 40 ml liter<sup>-1</sup> of lysis buffer (Tris 50 mM, NaCl 300 mM, imidazole 10 mM, pH 8) supplemented with 0,25 mg ml<sup>-1</sup> lysozyme, 20 ug ml<sup>-1</sup> DNase, 20 mM MgSO<sub>4</sub> and antiproteases (Complete EDTA-free antiprotases, Roche) and frozen at -80°C. After thawing and sonication, lysates were cleared by centrifugation (30 min at 12,000×g. The proteins were purified on a Pharmacia AKTA FPLC by nickel affinity chromatography (His-Trap 5 ml column, GE Healthcare) using a step-gradient of imidazole followed by a preparative Superdex 200 gel filtration in 50 mM Tris, 50 mM NaCl, pH 8. Proteins used in the crosslinking experiment were purified according to the recommendations in the QIAexpressionist manual (Qiagen).

Concentrations of the purified proteins were determined using Advanced Protein Assay Reagent (Fluka) and NanoDrop 1000 (Thermo Scientific). A<sub>280</sub> values were corrected for differences in absorption coefficient due to amino acid composition using the ProtParam tool (<http://www.expasy.org/tools/protparam.html>).

**Size exclusion chromatography (SEC) with On-line Multiangle Laser Light Scattering, Absorbance, and Refractive Index (MALS/UV/RI) Detectors.** SEC was carried out on an Alliance 2695 HPLC system (Waters) using a Superose S12 column eluted with 50 mM Tris and 50 mM NaCl at pH 8.0 at a flow of 0.5 ml min<sup>-1</sup>. Detection was performed using a triple-angle light scattering detector (Mini-DAWN<sup>TM</sup> TREOS, Wyatt Technology), a quasi-elastic light scattering instrument (Dynapro<sup>TM</sup>, Wyatt Technology), and a differential refractometer (Optilab\_rEX, Wyatt Technology). Molecular weight and hydrodynamic radius determination was performed by the ASTRA V software (Wyatt Technology) using a dn/dc value of 0.185 ml g<sup>-1</sup>. Proteins were loaded at a final concentration of 0.22 mM and 0.33 mM for AbiV and SaV, respectively.

**Cross-linking of AbiV and SaV.** A glutaraldehyde (GA) cross-linking experiment was performed as described previously<sup>53</sup>. Purified AbiV and SaV were diluted both separately and mixed in 50 mM Hepes, pH 6.2 to a final concentration of 17.6 μM and 32.9 μM, respectively for the two proteins. At time zero, glutaraldehyde was added to the protein samples to a final concentration of 0.05 % and the samples were incubated at room temperature. Aliquots were removed at time T-5



(before GA addition), T1 and T30 and immediately mixed with SDS-PAGE sample loading buffer followed by heating (99°C, 2 min) and storing on ice until analyzed on SDS-PAGE gels. The cross-linked proteins were analyzed on two identical SDS-PAGE gels of which one was Coomassie stained for identification of all protein bands and the other was analyzed by western blotting for identification of protein complexes containing SaV. The molecular weight of the protein bands was estimated using the marker Precision Plus Protein Dual Color Standard (Biorad).

**Fluorescence quenching experiments.** Fluorescence experiments were carried out on a Varian Eclipse spectrofluorimeter using a quartz cuvette in a right-angle configuration as previously described<sup>60</sup>. Briefly, the light path was 1.0 and 0.4 cm for the excitation and emission, respectively. The interaction of AbiV with SaV was monitored by recording the quenching of the intrinsic SaV protein fluorescence upon addition of AbiV aliquots which does not have an intrinsic fluorescence or absorbance at 295 nm. The excitation wavelength was 295 nm, and emission spectra were recorded in the range of 320 to 400 nm. The excitation slit was 5 nm while the emission slit was 10 nm for a protein concentration of 2 µM. A moving-average smoothing procedure was applied, with a window of 5 nm. Titrations were carried out at room temperature with 2 µM protein in 10 mM Tris Buffer, 50 mM NaCl, pH 8. No correction of the fluorescence at the maximum level (341 nm) was needed since the fluorescence and absorbance levels of the buffer and the quencher protein were negligible. The affinity was estimated by plotting the decrease of fluorescence intensity at the emission maximum as  $100 - (I_i - I_{\min})/(I_0 - I_{\min}) \times 100$  against the quencher concentration;  $I_0$  is the maximum of fluorescence intensity of the protein alone,  $I_i$  is the fluorescence intensity after the addition of quencher (i), and  $I_{\min}$  is the fluorescence intensity at saturating concentration of quencher. The  $K_d$  values were estimated using Prism 3.02 (GraphPad Software Inc.) by nonlinear regression for a double binding site with the equation  $Y = [(B_{\max 1} \cdot X)/(K_{d1} \cdot X)] + [(B_{\max 2} \cdot X)/(K_{d2} \cdot X)]$ , where  $B_{\max}$  is the maximal binding and  $K_d$  is the concentration of ligand required to reach half-maximal binding.

**Western blotting.** Primary SaV antibody was produced by customhybridoma.com, while primary antibodies against ORF11 and ORF16 were produced by C. Cambillau.

Proteins were separated by SDS-PAGE electrophoresis<sup>41</sup> using 11 % gels for ORF11 and ORF16 and 9 % gels for SaV. The proteins were then electroblotted (200 mA for 1 h) onto a PVDF membrane (Hybond P) by using a 20% ethanol solution containing 25 mM Tris and 192 mM

glycine pH 8.3 as transfer buffer in a Trans-Blot SD apparatus (BioRad). The membranes were subsequently treated with blocking buffer (5 % non-fat dry milk (NFDM) in phosphate buffered saline supplemented with 0.1 % tween-20 (PBS-T)) for 1 h on an orbital shaker and then treated (1 h, shaking) with primary antibody diluted in blocking buffer. For SaV, ORF11, and ORF16 primary antibodies, the following dilutions were used: 1:75,000; 1:100,000 and 1:25,000, respectively. After three washes with PBS-T the membrane was incubated (1 h, shaking) with secondary antibody (anti-rabbit IgG alkaline phosphatase linked whole antibody, Amersham) diluted 1:100,000 in blocking buffer. This was followed by three washes with PBS-T and 10 min equilibration in PBS before the membrane was treated with the ECF-substrate (Amersham) according to the manufacturers' instructions. The protein bands were then visualized using a STORM 860 scanner in the blue excitation (450 nm) fluorescence acquisition mode. Quantification of the fluorescent signal was performed using the software ImageQuant TL v.2003.03 (Amersham).

**Oligonucleotides and <sup>32</sup>P-labelling.** Oligonucleotides used for probing blotted mRNA were designed ensuring a high specificity for the target. These oligonucleotides were labelled with [<sup>32</sup>P]ATP (Easytides, Perkin Elmer) using polynucleotide kinase (Roche) and subsequent purification with NucAway spin columns (Ambion). Labelling efficiency was determined by quantification of 5 µl labelled probe using a Packard Instant Imager.

**RNA isolation and dot blotting.** Cell pellets were resuspended and incubated (37°C, 15 min) in 0.5 M sucrose with 60 mg ml<sup>-1</sup> lysozyme before being mixed with 1 ml TRIzol reagent (Invitrogen). Total RNA was then isolated according to the manufacturers' instructions and the samples were treated with the DNase based TURBO DNA-free kit (Applied Biosystems) before storing at -80°C. Immediately before use, the RNA samples were thawed and 0.5 µg RNA was added to 0.5 ml of a denaturing solution containing 10 mM NaOH and 1 mM EDTA. The RNA samples were blotted onto Zeta-probe nylon membranes (BioRad) by use of a Bio-Dot SF slot blot apparatus (BioRad). After a brief rinse in 2× SSC<sup>54</sup> plus 0.1% sodium dodecyl sulfate (SDS) for 1 min at room temperature, the membrane was air dried for 10 min and fixed by exposure to UV-light for 2 minutes on each side. Membranes were prehybridized for minimum 2 h in UltraHyb-Oligo hybridization buffer (Ambion) before <sup>32</sup>P-labeled riboprobe was added. After hybridization overnight at 42°C, the membranes were washed three times at 42°C in 2× SSC + 0.5 % SDS for 30 min before being air-dried. The amount of radioactivity retained by the riboprobes on the

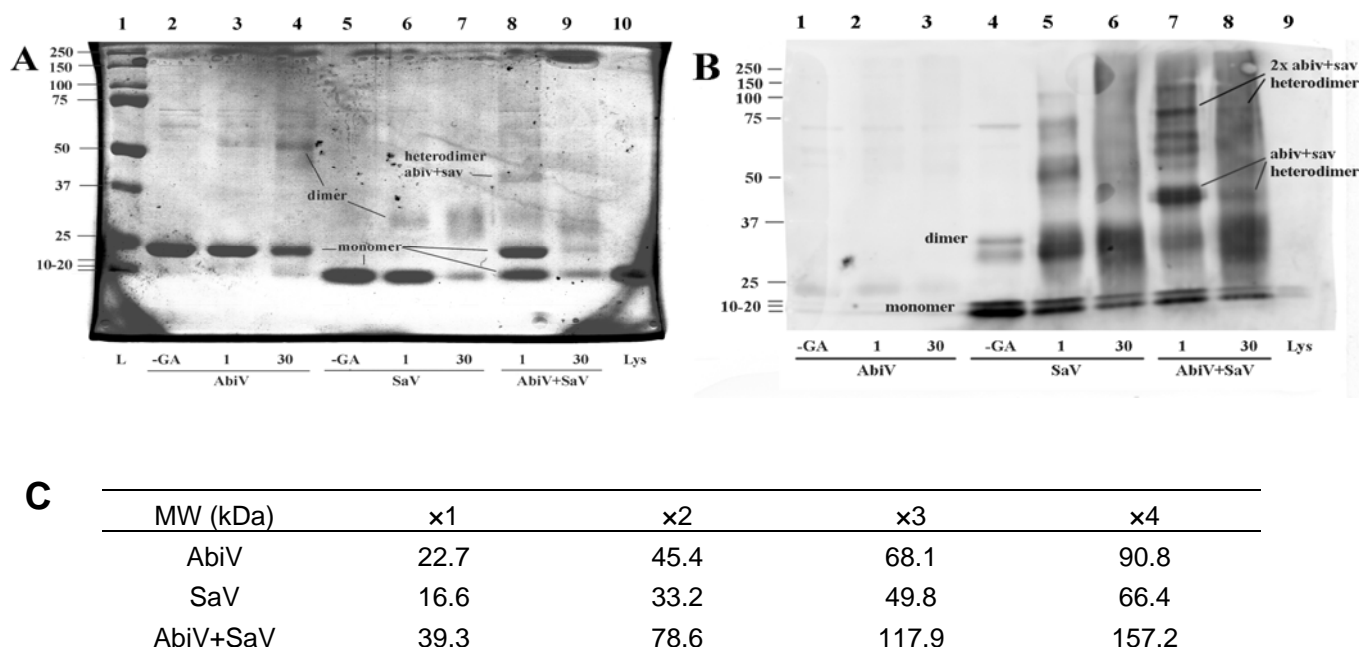
membranes was measured by overnight exposure of Storage Phosphor Screens (Amersham) and subsequent detection in a STORM 860 scanner in storage phosphor acquisition mode. Quantification of the radioactive signal was performed using the software ImageQuant TL v.2003.03 (Amersham).

**Radioactive determination of total RNA and protein synthesis.** For labelling of RNA, 2.3 ml of culture was mixed with 2  $\mu$ l of [ $^{14}$ C]uridine (50  $\mu$ Ci  $\text{ml}^{-1}$ ) and 6  $\mu$ l of 10 mM uridine to a final uridine concentration of 55  $\mu$ M. Labelling of proteins was done by adding 5  $\mu$ l of [ $^{35}$ S]methionine (15 mCi  $\text{ml}^{-1}$ ) to 1.7 ml of culture (the concentration of methionine in the SA medium was reduced to 5  $\mu$ g  $\text{ml}^{-1}$ ). Samples (200  $\mu$ l for RNA and 150  $\mu$ l for protein) were taken from the labelled cultures at 5 min intervals, transferred to a tube with 3 ml of cold 5% trichloroacetic acid (TCA) and put on ice for 1 to 1.5 h. Macromolecules were collected on a membrane filter (0.45- $\mu$ m pore size; Schleicher & Schuell, Dassel/Reliehausen, Germany), washed twice with cold 5% TCA and once with boiling water, and left to air dry. The radioactivity on the filters was counted in a Packard Instant Imager<sup>36</sup>.

**Phage DNA replication.** Replication of phage DNA was followed in a time course experiment as described previously<sup>28,31,36</sup>. Briefly, total DNA from *L. lactis* cells was isolated at regular intervals from cultures either infected with phage sk1 or phage c2 or from non-infected cultures. Total DNA was digested with EcoRV, heated (65°C, 10 min), and analyzed by eletrophoresis on an agarose gel.

## RESULTS

**AbiV and SaV interact by direct protein-protein interaction.** To determine if AbiV and SaV were interacting directly to form complexes, we have used three different approaches: cross-linking assays, SEC-MALS/UV/RI and fluorescence quenching experiments. First, we made an *in vitro* cross-linking experiment with purified AbiV and SaV. The proteins were assayed at room temperature separately and also mixed in the molecular ratio AbiV:SaV = 1:1. Samples were analyzed using coomassie staining (Fig. 1A) and SaV specific western blotting (Fig. 1B). On figure 1A, the AbiV monomer (22.7 kDa, Fig. 1C) is visible along with faint high-MW band in lane 2 before GA addition. The monomer band is fading in the lane 3 and 4 (T1 and T30, respectively) concomitantly with the appearance of a band around 50 kDa, which probably represents an AbiV dimer. The same pattern is observed for SaV (Fig. 1A, lanes 5-7). In the AbiV-SaV mixed condition (Fig. 1A, lanes 8-9) both monomers and the SaV dimer are observed along with a distinct band of about 40 kDa, which corresponds to an AbiV-SaV heterodimer (39.3 kDa, Fig. 1C). Lysozyme (monomer, ca. 14.5 kDa) was treated for 30 min with 0.05 % GA and the absence of cross-linking of this protein serves as a negative control (Fig. 1A, lane 10).

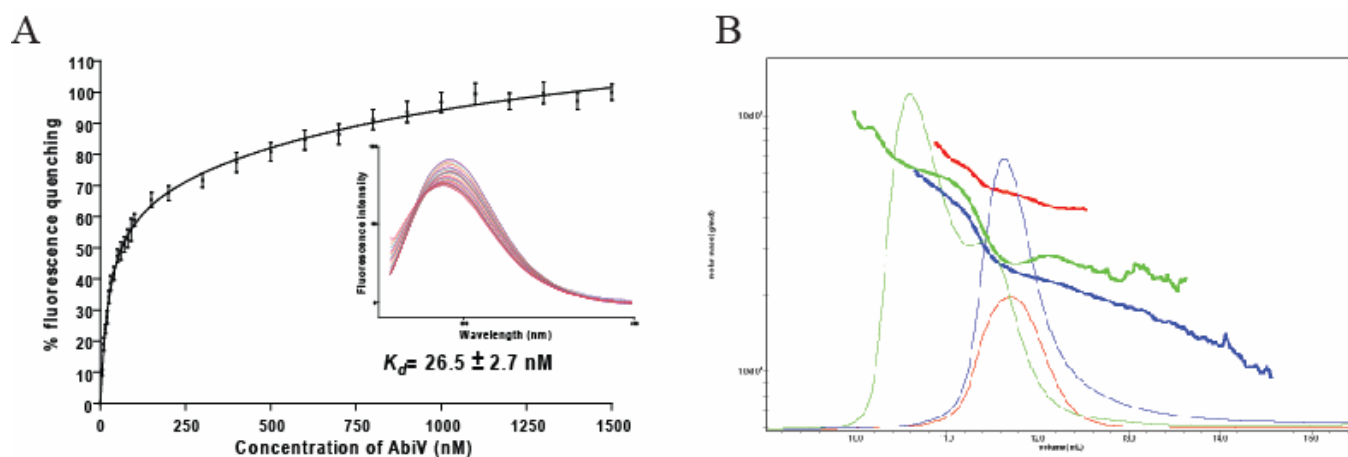


**Fig. 1.** Cross-linking experiment with AbiV and SaV using Coomassie stained SDS-PAGE gel (A) and western blot with detection of SaV (B). The theoretical molecular weights (kDa) of different hypothetical multimeric combinations of AbiV and SaV are presented in (C). Molecular weight was determined using Precision Plus Protein Standard (Biorad). Lane designations are: L is ladder, -GA is before addition of glutaraldehyde, 1 and 30 represents minutes and Lys is lysozyme. Numbers above gel represent lane numbers.

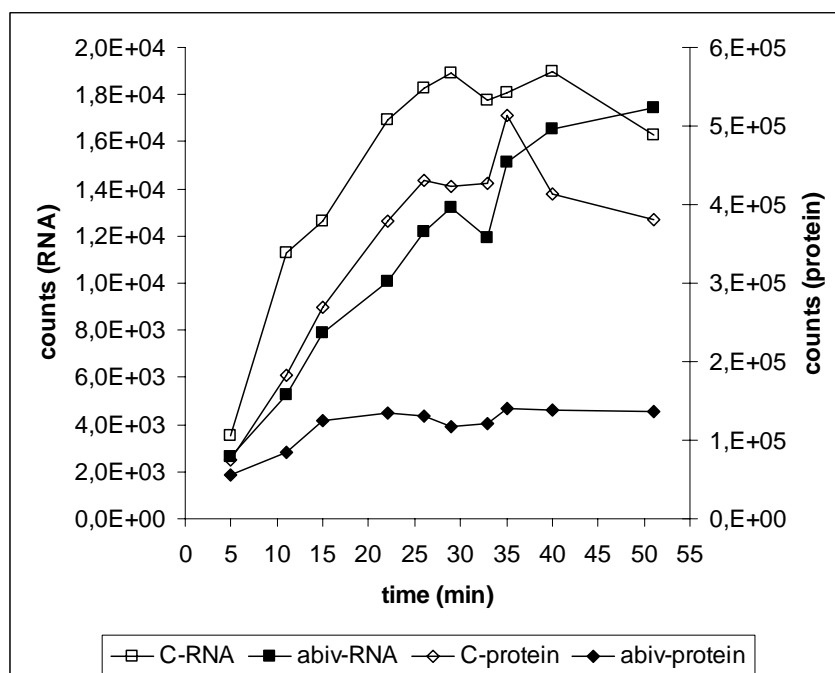
Lane 4 on the Western Blot (Fig. 1B) shows SaV before cross-linking including distinct bands of ca 15 and 30 kDa. These bands may represent monomers and dimers. An additional band of ca 50 kDa that would correspond to a SaV trimer is observed after addition of GA. In the AbiV-SaV mix, the SaV monomer and dimer are observed but also a very distinct band of ca 40 kDa (Fig. 1B, lane 7). This protein contains SaV and the molecular weight indicates that it is a hetero-dimer consisting of a SaV monomer and an AbiV monomer. Several other bands are observed including a band of ca 80 kDa that possibly represents the cross-linking of a SaV dimer and an AbiV dimer. In addition to suggesting AbiV-SaV interaction, the above data supports our previous observations that both AbiV and SaV are native dimers<sup>28,29</sup>.

To determine more precisely the sizes of the AbiV and SaV homodimers, and the size of the complex AbiV/SaV, we used SEC-MALS/UV/RI. SEC is a simple way to estimate the size of a protein complex based on a calibration curve obtained with a protein standard. However, the elution position is not only related to the molecular mass but is also influenced by the protein shape and the affinity of the protein with the matrix used in the column. Therefore, to obtain a better estimate of the molecular mass of the protein, it is possible to combine SEC with on-line multiangle laser light scattering and refractrometry (MALS/UV/RI). The molecular mass measured this way is more accurate since the measure is independent of the position of elution. AbiV and SaV were subjected to mass and size analysis separately and together. The MALS/UV/RI analysis gave a measured mass of 47,550 Da and 36,000 Da for AbiV and SaV, respectively, which indicate that both proteins form homodimers (Fig 1C and Fig. 2B). When both proteins are injected together, the chromatogram shows a single peak. The measured mass (71,410 Da) corresponds to a complex consisting of AbiV<sub>2</sub>SaV<sub>2</sub> (75,756 Da, Fig. 2B). The hydrodynamic rayon ( $R_h$ ) of AbiV and SaV is 3.0 nm and 3.2 nm, respectively, while the  $R_h$  of the complex is 4.0.

The dynamic of the AbiV-SaV interaction was investigated using fluorescence quenching. Since AbiV has no intrinsic fluorescence it was possible to measure the dynamic of the complex association by adding aliquots of AbiV to a sample of SaV. Addition of AbiV to SaV leads to the reduction of emission of Trp fluorescence when excited at 295 nm. A good fit between the experimental data and the theoretical curve could be obtained when two binding sites were taken into account (Fig. 2A). The  $K_d$  value was  $26.5 \pm 2.7$  nM.



**Fig. 2.** Fluorescence quenching assay fitting curve using a nonlinear regression with a double binding site equation (A). The insertion represents the plot of the fluorescence emission in function of the wavelength (nm) of acquisition. SEC-MALS/UV/RI analysis (B). The abscissa indicates the volume scale of the HPLC injection. The ordinate indicates the molar mass in  $\text{g mol}^{-1}$  (Da). AbiV data is represented in red, SaV data is in blue and the complex AbiV/SaV is in green. The resolution of Fig. 2B is low. Data for obtaining higher resolution was not available at the time of submission of this thesis.



**Fig 3:** Total synthesis of RNA (squares) and protein (diamonds) in AbiV<sup>+</sup> cells (closed symbols) and AbiV<sup>-</sup> cells (open symbols) during lytic cycle of phage p2. RNA and protein synthesis were determined as total incorporation of [<sup>14</sup>C]-uridine and [<sup>35</sup>S]-methionine, respectively

### **The AbiV-SaV interaction affects total synthesis of RNA and protein during phage infection.**

We previously demonstrated that DNA of phage p2 (936 species) is replicated during infection of cells containing AbiV but that the infection is aborted prior to packaging of the phage DNA<sup>28</sup>. To get a more detailed picture of the effects of AbiV on synthesis of macromolecules during phage infection, we conducted an experiment in which we measured total synthesis of RNA and protein in both AbiV<sup>+</sup> and AbiV<sup>-</sup> cells during infection with phage p2. Exponentially growing cultures of JH-20 (AbiV<sup>+</sup>) and JH-54 (AbiV<sup>-</sup>) were infected with phage p2 (MOI = 5) at OD<sub>600</sub> = 0.5 while a non-infected JH-20 culture served as control. Aliquots of all cultures were immediately added to solutions containing <sup>14</sup>C-uridine (RNA synthesis) or <sup>35</sup>S-methionine (protein synthesis) and samples were taken at 5 minute intervals for determination of total RNA or protein synthesis. Lysis of the sensitive AbiV<sup>-</sup> culture was observed 28 minutes after infection.

Compared to the non-infected control, addition of phages (in both AbiV<sup>+</sup> and AbiV<sup>-</sup> cells) caused an almost immediate and drastic decline in RNA and protein synthesis indicating that production of host factors are shut down by the phage quickly after infection (data not shown). RNA and protein synthesis in the infected AbiV<sup>-</sup> cells (Fig. 3) increased until T28 and T26, respectively when synthesis stopped. This coincided with complete lysis of the culture 28 min after infection. In the AbiV<sup>+</sup> cells RNA was produced though at a reduced rate compared to the AbiV<sup>-</sup> culture (Fig. 3), and synthesis continued throughout the experiment though it levelled off just before termination of the experiment. The continued synthesis of RNA in this culture was possibly due to the absence of cell lysis and this indicates that despite the slight inhibition of RNA synthesis, the primary interference of AbiV with the lytic cycle is not at the level of transcription. On the contrary, protein synthesis was severely inhibited in the AbiV<sup>+</sup> culture, which compared to the AbiV<sup>-</sup> culture showed significantly slower protein synthesis the first 15 min of the infection, at which time protein synthesis stopped completely (Fig. 3). The above data shows that despite a significant production of RNA, no protein synthesis was observed which indicates that the mechanism of AbiV involves inhibition of translation.

**AbiV affects transcription of middle and late phage genes.** Knowing that the AbiV mechanism probably inhibits phage protein synthesis we wanted to address more specifically which part of the phage lytic cycle is targeted by AbiV. In another phage infection experiment, JH-20 (AbiV<sup>+</sup>) and JH-54 (AbiV<sup>-</sup>) were grown exponentially to OD<sub>600</sub> = 0.5 when the cells were concentrated  $\times 10$  by spinning and resuspending in fresh medium. After infection with phage p2 (MOI = 5) samples for

isolation of RNA and protein were taken at 5 min intervals by quick-spin and freezing of the pellet in -80°C liquid ethanol. Lysis of the sensitive AbiV<sup>-</sup> culture occurred 29 minutes after infection and the lysis was accompanied by a rise in phage titer corresponding to a burst size of ca. 50 pfu.

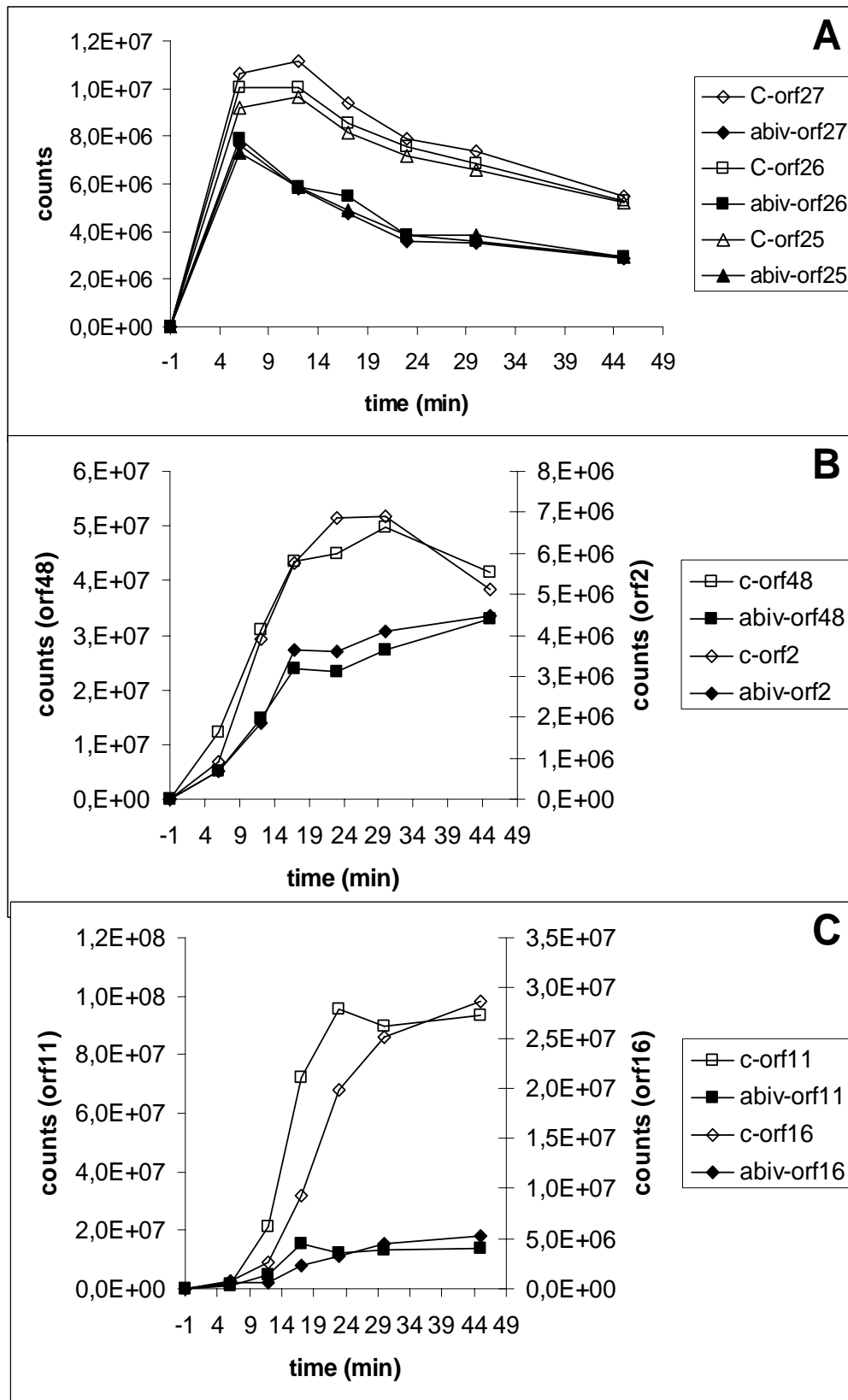
To quantify the transcription level at different time-points during the lytic cycle of the phage, several radioactively labelled oligonucleotide probes covering the early, middle and late regions of p2 were used to probe the RNA in a dot blot assay. In the early region, three genes (*orf27*, *orf26* (*sav*), and *orf25*) were analyzed. In the AbiV<sup>-</sup> cells they all reached the same level of transcription which peaked between 6 and 12 minutes after which they gradually decreased throughout the rest of the experiment (Fig. 4A). The similar transcription levels of *orf27* and *sav* is due to their position in the same operon. In the AbiV<sup>+</sup> cells, transcription levels were also equal among the three genes. The highest level was observed after 6 minutes after which transcription gradually decreased as for the AbiV<sup>-</sup> cells. In these cells transcription level of the early genes at T6 was 76 % of the level in the AbiV<sup>-</sup> cells. At T12 and throughout the experiment, the level was ca 60 % of the AbiV<sup>-</sup> cells.

Two genes (*orf48* and *orf2*) had almost identical transcription patterns (Fig. 4B). *Orf48* is part of the p2 putative middle operon whereas *orf2* is part of the putative late operon. In the AbiV<sup>-</sup> cells both transcripts increased until T23 whereas in the AbiV<sup>+</sup> cells they levelled off at T17 reaching ca 50 % of the level in the AbiV<sup>-</sup> cells.

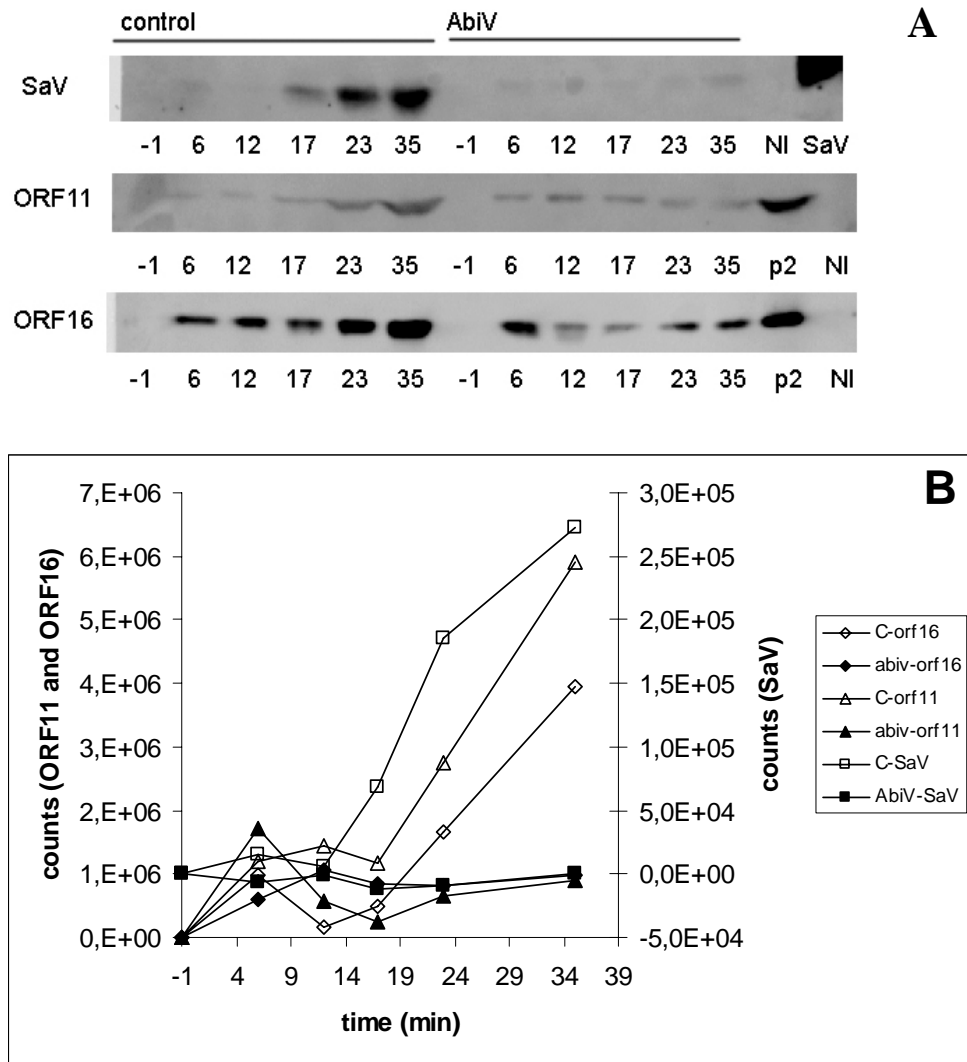
The late transcripts (*orf11* and *orf16*) started to increase in the AbiV<sup>-</sup> cells between T6 and T13 (Fig. 4C). *Orf16* was slightly delayed compared to *orf11* because of its location ca. 5.5 kb downstream of *orf11*. For both genes, transcription in the AbiV<sup>+</sup> cells reached only ca 10 % of the level in the AbiV<sup>-</sup> cells.

The above transcription data shows that mRNA is produced in the presence of AbiV thus supporting the data demonstrating synthesis of total RNA in the presence of AbiV. However, the transcription data also shows that there is a differentiation of the synthesized mRNA which is linked to the timing of transcription. Thus early transcripts are only little affected (75 % to 60 % of wild type level), whereas middle transcripts are slightly more affected (50 % of wild type level) and late transcripts are almost completely inhibited (10 % of wild type level).





**Fig 4:** Transcription of early (A), middle (B) and late (C) genes during infection of AbiV<sup>+</sup> cells (closed symbols) and AbiV<sup>-</sup> cells (open symbols) with phage p2



**Fig 5.** Translation of phage proteins SaV, ORF11 and ORF16 during infection with phage p2. Western blot (A) shows bands in AbiV<sup>-</sup> cells (control) and AbiV<sup>+</sup> cells (AbiV) at different timepoints (-1 to 35 minutes). NI is non-infected, SaV is purified SaV and p2 is isolated structural proteins from phage p2. The levels of expression are shown in (B) where open and closed symbols represent AbiV<sup>-</sup> and AbiV<sup>+</sup> cells, respectively

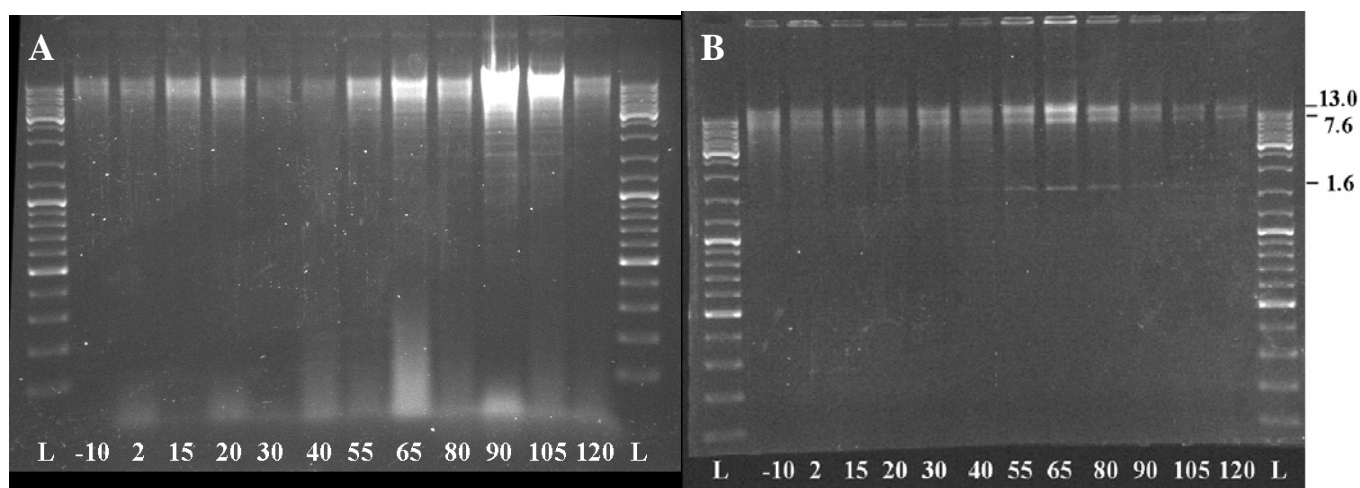
**AbiV inhibits translation of phage proteins.** Total protein was isolated from samples taken simultaneously with the RNA samples. Using western blotting and antibodies specific for SaV, ORF11 and ORF16 we were able to follow the level of translation during the p2 lytic cycle. SaV is a non-structural protein located in the early expressed region of the phage while ORF11 is the major capsid protein (MCP) and ORF16 probably is associated with the tail. Both are late structural proteins.

In the AbiV<sup>-</sup> cells SaV translation increased from T12 and continued to rise throughout the experiment (Fig. 5). No translated SaV could be detected in the AbiV<sup>+</sup> cells at any time during the

experiment. The same pattern was observed for the late proteins ORF11 and ORF16 which both increased from T17 in the AbiV<sup>-</sup> cells. Contrary to the SaV samples a basic level of protein was observed in the ORF11 and ORF16 samples from AbiV<sup>+</sup> cells, which was due to the initial amount of phages added in the experiment (MOI = 5). Since ORF11 and ORF16 are structural proteins these proteins were detected at a basic and stable level in the AbiV<sup>+</sup> cells (Fig. 5A). The observed timing of expression of the three proteins was expected due to their position on the phage genome. Thus SaV was expressed before ORF11 which was expressed before ORF16 (Fig. 5B). Expression of SaV is expected to start immediately after transcription so the observed delay (expression increasing from T12) was probably due to lack of sensitivity of the western blotting.

The western blots showed that translation of both early and late phage proteins were severely inhibited. For ORF11 and ORF16 this can be explained by the low level of transcribed mRNA but in the case of SaV, the gene is transcribed almost at wild type level (Fig. 4A). The results therefore indicate that AbiV in combination with SaV inhibit translation of phage p2 proteins early in the lytic cycle and possibly by a direct protein interaction between the two proteins.

**Inhibition of DNA replication in phage c2.** We previously demonstrated that DNA of the 936-phage p2 was replicated in AbiV<sup>+</sup> cells during a phage infection experiment but that the replicated DNA only existed in the concatemeric form<sup>28</sup>. We observed the same pattern with the 936 phage sk1 (data not shown). An identical experiment was conducted in which phage sk1 was substituted with phage c2 to infect the same host (*L. lactis* MG1363) with and without AbiV. In the AbiV<sup>-</sup> cells, replicated c2 DNA was observed at T55 and starting to decrease at T80 coinciding with lysis of the culture (Fig. 6B). The low MOI (ca. 1) used in this experiment probably caused the late lysis of the sensitive host. In the AbiV<sup>+</sup> cells the pattern of EcoRV digested c2 DNA could not be observed at any time during the experiment (Fig. 6A). The bands observed on the gel therefore represent the EcoRV digested bacterial chromosome. The data indicates that c2 DNA is not replicated in the presence of AbiV thereby demonstrating different phenotypic effects of AbiV on the lytic cycle of 936- and c2-phages.



**C**



**Fig. 6.** Phage DNA replication in AbiV<sup>+</sup> cells (A) and AbiV<sup>-</sup> cells (B) during lytic cycle of phage c2. Total DNA was isolated at different timepoints (-10 to 120 minutes) after infection and digested with EcoRV. Restriction maps of c2 digested with EcoRV is presented (C) where numbers indicate sizes of the restriction fragments. Numbers refer to the size of c2 DNA fragments after digestion and heat treatment. L is GeneRuler 100-10,000 DNA ladder (Fermentas)

## DISCUSSION

In recent studies we have isolated the lactococcal abortive infection mechanism AbiV<sup>28</sup> and also characterized the early transcribed phage protein SaV which is necessary for the abortive infection phenotype of the system<sup>29</sup>. In the present study we demonstrated a direct protein-protein interaction between AbiV and the translated phage gene product SaV using cross-linking, SEC-MALS/UV/RI and fluorescence quenching assays (Fig. 1+2). AbiV and SaV form a complex composed of 2 units of each protein. The strength of interaction was measured by fluorescence quenching assay and the analysis gave a  $K_d$  value of  $26.5 \pm 2.7$  nM. The interaction is significant and is, to our knowledge, the first demonstration of a direct interaction between an Abi-protein and an interacting phage protein. This data confirmed our previous observations that both AbiV<sup>28</sup> and SaV<sup>29</sup> form native dimers. We previously argued that the SaV polypeptide is involved in sensitivity to AbiV<sup>29</sup> and it is thus probable that the direct AbiV-SaV protein interaction is responsible for the Abi phenotype.

In order to elucidate the molecular effects of the AbiV-SaV interaction on the lytic cycle of 936 phages we used phage p2 as a model. Phage p2 is a very close relative to phage sk1 (GenBank acc.nr: [NC\\_001835](#)).

The transcription level of genes located in the early, middle and late region of phage p2 was analyzed with and without AbiV at different time points during a lytic cycle (Fig. 4). We observed an increasing effect of AbiV on transcription from early to middle and late genes which was most pronounced for transcripts in the late region. In the early region we observed a 25-40 % decrease of transcription in AbiV<sup>+</sup> cells compared to AbiV<sup>-</sup> cells. In the middle and late regions the decrease was 50 % and 90 % respectively. Previous transcription analyses of sk1 have revealed that early transcripts appear 2-5 minutes after infection whereas middle transcripts are observed after 7-10 minutes and late transcripts after 15 minutes<sup>9</sup>. In the AbiV<sup>-</sup> cells, early transcripts are decaying from T12 maybe due to a switch-off mechanism as observed in other phages<sup>9,20,52</sup> coinciding with the appearance of late transcripts 15 minutes after infection. In the AbiV<sup>+</sup> cells these transcripts are decaying earlier thus ending at a level which is 60 % of the AbiV<sup>-</sup> cells. These early transcripts are expected to be important primarily in the beginning of the lytic cycle where the effect of AbiV on transcription was small (25 % reduction at T6). Previously, half-life of mRNA in *L. lactis* has been determined to ca. 2 minutes<sup>62</sup>. Given this rate of mRNA decay, the transcription data suggests that there is a continuous mRNA production both in AbiV<sup>+</sup> and AbiV<sup>-</sup> cells. This was supported by

results from the total RNA synthesis experiment, in which, data was not influenced by mRNA stability (Fig. 3).

Contrary to RNA synthesis, a profound effect of AbiV was observed on protein synthesis in AbiV<sup>+</sup> cells compared to AbiV<sup>-</sup> cells. Total protein synthesis was severely inhibited from the beginning and ceased completely after 15 minutes of the lytic cycle. According to the *sk1* transcription data<sup>9</sup> this prevented translation of most middle transcripts (7-10 min after infection) and all late transcripts (15 min after infection). Western blotting and antibody identification of middle proteins was not performed but as expected, translation of the two late proteins ORF11 and ORF16 was completely inhibited by the AbiV mechanism (Fig. 5). Interestingly, translation of SaV could not be detected in the AbiV<sup>+</sup> cells though the *sav* transcript was observed in these cells (Fig. 4A). The Abi phenotype is supposed to involve a translated SaV protein<sup>29</sup> but since this could not be detected using western blotting, we suggest that very small amounts of SaV are needed to induce the Abi phenotype and that the western blotting method is not sensitive enough to detect this amount of SaV. SaV translation is thus almost completely inhibited by the AbiV mechanism while transcription of genes in the early region (including *sav*) is only minimally inhibited by AbiV. These observations strongly indicate that the target of the AbiV mechanism is to be found in the translation apparatus of the cell.

To account for the above observations we constructed the following hypothesis on the mode of action (MOA) of the AbiV mechanism: Upon translation, the SaV polypeptide interacts with AbiV to induce the Abi phenotype inhibiting the translational machinery of the cell. If only small amounts of SaV is needed for induction of the Abi mechanism, translation will be arrested early in the lytic cycle thereby also preventing further SaV translation, which is therefore only produced in sub-detectable amounts. Middle genes in *sk1* are transcribed from the P<sub>M</sub> promoter which needs activation most probably by an early translated phage protein<sup>3,10,39</sup>. Since translation is arrested early in the lytic cycle this activator is probably not fully expressed thereby causing the observed partial inhibition of middle transcripts. The transcription profile of *orf48* is remarkably similar to the profile of *orf2* (Fig. 4B). In the analysis of the phage *sk1* genome the homologue of *orf48* was assigned to the middle region whereas *orf2* was assigned to the late region<sup>10</sup>. In the same study a putative terminator structure was suggested to exist at nucleotide position 27,890 to 27,908 on the *sk1* genome (GenBank acc.nr: [NC\\_001835](#)). However this stem-loop structure is not succeeded by the oligo(T) sequence (T-stretch) which is crucial for intrinsic transcription termination<sup>27</sup>. It is therefore likely that the terminator is inefficient thereby causing read-through from the P<sub>M</sub> promoter

which was observed in both sk1<sup>9,27</sup> and p2 (this study). The late transcripts in sk1 were observed 15 minutes after infection whereas middle transcripts were observed after 7-10 minutes<sup>9</sup>, indicating that one of the four genes in the middle operon is an activator of late transcription<sup>9</sup>. In the present study such an activator would be translated at very low levels in the AbiV<sup>+</sup> cells due to the combined effect of inhibited transcription of the middle region and the translation inhibition by the Abi mechanism. The almost complete inhibition of the two late transcripts (*orf11* and *orf16*, Fig. 4C) could thus be explained by absence of activation.

Our data does not support a more detailed description of the AbiV-SaV mode of action. However, using the software RNABindR and BindN, we identified a putative RNA binding site at codon 81-96<sup>63</sup> or 81-113 of AbiV<sup>57,58</sup>. It could be speculated that this RNA binding site is not exposed in the native non-toxic form of AbiV<sup>28</sup>, but that activation by SaV causes a conformational change in which the RNA binding site is exposed. RNA binding by an active AbiV-SaV complex could therefore be part of the AbiV mode of action. However, further studies are needed to test this hypothesis.

DNA replication of phage p2 from the 936 species was previously demonstrated to occur in AbiV<sup>+</sup> cells<sup>28</sup>. While the same pattern was observed using the closely related 936 phage sk1 in the present study, we showed that DNA replication did not occur when the same host was infected with the prolate headed phage c2 (Fig. 6). Since the same host was used in the experiments, differences between the two phage types must account for the two observed phenotypes<sup>25</sup>. For some Abi mechanisms different modes of action have been observed against phages from the 936, c2, and P335 species<sup>11,16,56</sup>: AbiK allowed DNA replication of phage p2 (936-species) but not ul36 (P335-species)<sup>7</sup> and AbiG allowed DNA replication of c2 and 936 phages<sup>51</sup> but not of P335 phages<sup>56</sup>. We previously demonstrated that while the copy-number of AbiV apparently was not important for the Abi phenotype against the 936 phages p2 and sk1, protection against c2 infection required high expression of AbiV<sup>28</sup>. Such stronger Abi effects on 936 phages compared to c2 phages has been demonstrated for AbiA<sup>16</sup>, AbiG<sup>51</sup>, and AbiK<sup>22</sup> suggesting that c2 phages are generally better suited to bypass the Abi phenotype compared to 936-phages<sup>56</sup>. We previously demonstrated that like AbiA<sup>16</sup> and AbiK<sup>6</sup>, the phage genes involved in sensitivity to AbiV share only limited amino acid similarity between 936 and c2 phages<sup>29</sup>. However, a region of SaV which is conserved among the 936 and c2 species is probably involved in sensitivity to AbiV which could suggest a common mode of action of AbiV against the two phage species. If translation inhibition of phage proteins is the MOA of AbiV against both 936 and c2 phages it implies that the mechanism must act before

translation of the DNA polymerase (*orfs e5, e6, and e7*) in c2<sup>8,43</sup>. The DNA polymerase subunits are transcribed early in the lytic cycle from consensus *Lactococcus* promoters in both the 936 phages sk1 and p2 and phage c2. However, the *sav* homologue (*e11*) of c2 is transcribed from a promoter which is much stronger than the promoter from which the DNA polymerase is transcribed<sup>42</sup>. This strong promoter was previously compared to the strong promoters from phage T5<sup>26,42</sup>. It has perfect consensus and strong conservation downstream of the start nucleotide and it was suggested to be among the strongest lactococcal promoters identified<sup>26,42</sup>. Transcription of the *sav* homologue of c2 from such a strong promoter could activate AbiV and thus the Abi phenotype before transcription and translation of the DNA polymerase which is being transcribed from a much weaker promoter<sup>42</sup>. In the analyzed 936 phages such differentiation between the promoters of *sav* and the DNA polymerase was not observed.

In conclusion, we have analyzed the interaction between the novel Abi mechanism AbiV<sup>28</sup> and the phage p2 protein SaV which is essential for the function of AbiV<sup>29</sup>. The proteins interact directly forming an active complex that might involve an exposed RNA binding site in AbiV. Ongoing work is aimed at revealing such structural information. The Abi mechanism was demonstrated to inhibit protein synthesis in phage p2 at an early stage of the lytic cycle while RNA synthesis was primarily inhibited for late mRNA transcripts probably due to absence of activation of a late promoter. We propose a hypothesis for the mode of action of AbiV against 936 phages which could also explain the different phenotypic effect of AbiV on the lytic cycle of phage c2.

## ACKNOWLEDGMENTS

This work was funded in part by a graduate scholarship from the Technical University of Denmark to JH and a strategic grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada to SM.



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# **MANUSCRIPT IV and V**



# Industrial applications of the novel lactococcal abortive infection mechanism AbiV

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## ABSTRACT

The lactococcal abortive infection mechanism AbiV is silent on the chromosome of *Lactococcus lactis* subsp. *cremoris* MG1363. Spontaneous phage resistant mutants were analyzed by reverse transcriptase PCR (RT-PCR) and demonstrated to express AbiV. The expression might be related to point mutations upstream of *abiV*. Conjugal transfer of *abiV* was demonstrated between two lactococcal strains by chromosomal transfer, thereby demonstrating, to our knowledge, the first conjugal transfer of a chromosomally encoded phage resistance mechanism.



## INTRODUCTION

Industrial milk fermentation is a world wide industry which is dependent on well characterized metabolic features of industrial starter strains <sup>7</sup>. Phage attacks, primarily by the three species 936, c2, and P335 <sup>33</sup>, leading to fermentation failure is a persistent problem for the dairy industry <sup>10</sup> despite decades of focused research on lactococcal phages <sup>7,33</sup>. A number of natural defense systems exist in *Lactococcus lactis* including adsorption inhibition <sup>15,26</sup> injection inhibition <sup>31</sup>, R/M systems <sup>1,15</sup>, and abortive infection (Abi) mechanisms <sup>5</sup>. These mechanisms have been used intensively in a relatively small number of industrial starter strains <sup>9</sup>, which has favored the emergence of phage mutants that are insensitive to the applied anti-phage barriers <sup>10,15,33</sup>. This forces the dairy industry to constantly finding new ways to protect their starter cultures against phage attacks. Engineered phage resistance has been suggested as a supplement to the natural phage resistance mechanisms <sup>7,11,12,23,32,27,31,8</sup>. However, these mechanisms have only limited efficiency against a narrow range of sensitive phages <sup>26</sup> and furthermore, industries using genetically modified organisms are facing obstacles as legal limitations in many countries <sup>15,33</sup> as well as consumer skepticism <sup>9,15,47</sup>. To protect their strains, the dairy industry is therefore dependent on isolation of novel natural phage resistance barriers that can be transferred to industrial starter strains without the use of genetic manipulation. Most natural R/M and Abi systems are plasmid encoded <sup>2,3,5,13,14,16,24,36,46</sup>. Many of these plasmids are conjugative and the concomitant mobility of the phage resistance mechanisms has been utilized by the dairy industry to create phage resistant starter cultures <sup>1,7,22,33,35,39,42</sup>. However, some phage resistance mechanisms are chromosomally encoded and the industrial application of these anti-phage barriers is dependent on the ability to transfer the gene(s) encoding the phage resistance mechanism to the desired industrial production strains. Contrary to conjugal transfer of plasmid encoded phage resistance mechanisms which has been widely exploited by the dairy industry, the conjugal transfer of chromosomally encoded phage resistance mechanisms has to our knowledge not been demonstrated.

Recently, we isolated and characterized AbiV, a novel Abi mechanism which is silent on the chromosome of *L. lactis* subsp. *cremoris* MG1363 <sup>20</sup>.

Here, we report phage resistant mutants of a *L. lactis* MG1363 derivative that spontaneously has mutated to express AbiV. Furthermore, we demonstrate that *abiV* can be transferred between lactococcal strains by conjugational chromosomal transfer and subsequent recombination events.

## RESULTS AND DISCUSSION

**Isolation of mutants of *L. lactis* MB112 spontaneously expressing AbiV.** The lactococcal phage abortive infection mechanism AbiV is located on the chromosome of *L. lactis* MG1363 among remnants of mobile genetic elements in a region referred to as an “integration hot spot”<sup>48</sup>. AbiV protects the cell against 936-like and c2-like phages when expressed in an expression vector while the level of expression in wild type *L. lactis* MG1363 is not sufficient to confer phage resistance<sup>20</sup>. To investigate if *L. lactis* MG1363 could mutate spontaneously to express AbiV, we isolated mutants that could grow in the presence of the lytic phage sk1 (all bacterial strains and phages used in the present study are listed in Table 1). Ten independent cultures of exponentially growing *L. lactis* MB112 (30°C in M17<sup>45</sup> supplemented with 0.5 % glucose) were mixed with the lytic phage sk1 (MOI > 1) in presence of 10 mM CaCl<sub>2</sub> and incubated 10 min at room temperature before plating and incubation at 36°C overnight. Mutants that spontaneously had gained resistance to sk1 were observed with a frequency of ca 10<sup>-8</sup>. Fifty-six single colonies picked randomly among the ten independent cultures were purified and cross-streaked<sup>40</sup> against phages sk1, p2, 712 and the AbiV insensitive mutant p2.1<sup>21</sup>. A mutant expressing AbiV is expected to be resistant to sk1 and p2 but sensitive to 712 and p2.1 due to the absence of the early transcribed gene *sav* in the two latter species (Table 1)<sup>21</sup>.

**TABLE 1.** List of bacteria and phages used in the study.

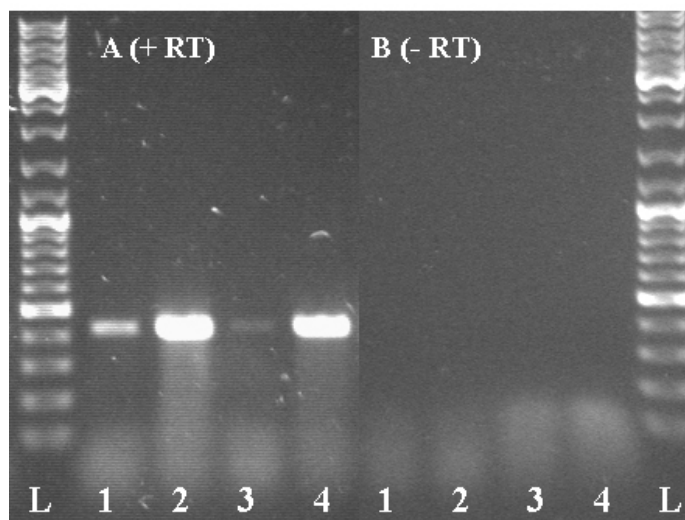
Bacterial strain or phage	Characteristics	Source
<b><i>L. lactis</i></b>		
MB112	<i>L. lactis</i> subsp. <i>cremoris</i> MG1363, $\Delta upp$ , host for phages p2, sk1, 712, and p2.1; FU <sup>R</sup>	30
MG1614	<i>L. lactis</i> MG1363 derivative, host for phages p2, sk1, 712, p2.1; Rif <sup>R</sup> , Str <sup>R</sup>	18
JH-20	MB112 (pJH2); Cam <sup>R</sup> , AbiV <sup>+</sup>	20
JH-32	MB112 (pGhost9::ISS1 insert upstream of <i>abiV</i> on chromosome); Erm <sup>R</sup> , FU <sup>R</sup> , AbiV <sup>+</sup>	20
JH-54	MB112 (pLC5), Cam <sup>R</sup> , AbiV <sup>-</sup>	20
JH-80	MB112, spontaneous resistant to sk1 and p2, AbiV <sup>+</sup>	This study
JH-83	MG1614 (R), JH-32 (D) transconjugant, FU <sup>S</sup> , Erm <sup>R</sup> , Str <sup>R</sup> , Rif <sup>R</sup> , p2 <sup>R</sup> , p2.1 <sup>S</sup> , sk1 <sup>R</sup> , 712 <sup>S</sup>	This study
<b>Phages</b>		
p2	Small isometric headed, 936 species, <i>sav</i> <sup>+</sup>	34
sk1	Small isometric headed, 936 species, <i>sav</i> <sup>+</sup>	4
712	Small isometric headed, 936 species, <i>sav</i> <sup>-</sup>	29
p2.1	Small isometric headed, 936 species, mutations and deletion in <i>sav</i> , <i>sav</i> <sup>-</sup>	21
Cam <sup>R</sup> , chloramphenicol resistance (5 µg ml <sup>-1</sup> ); Erm <sup>R</sup> , Erythromycin resistance (3 µg ml <sup>-1</sup> ); FU <sup>R</sup> , Fluorouracil resistance (0.3 µg ml <sup>-1</sup> ); Rif <sup>R</sup> , Rifampicin resistance (100 µg ml <sup>-1</sup> ); Str <sup>R</sup> , Streptomycin resistance (200 µg ml <sup>-1</sup> ); AbiV <sup>+</sup> , AbiV phage resistance phenotype; AbiV <sup>-</sup> , AbiV phage sensitive phenotype; p2 <sup>R</sup> , resistant to phage p2; p2.1 <sup>S</sup> , sensitive to phage p2.1; sk1 <sup>R</sup> , resistant to phage sk1; 712 <sup>S</sup> , sensitive to phage 712; , <i>sav</i> <sup>+</sup> , functional <i>sav</i> gene; <i>sav</i> <sup>-</sup> , absent or unfunctional <i>sav</i> gene.		

Of the fifty-six initial mutants, EOP values were obtained for eight possible candidates using the phages p2, 712, and p2.1. Phage p2 has an efficiency of plaquing<sup>41</sup> (EOP) of ca  $10^{-4}$  on cells harboring AbiV, and one mutant (JH-80) revealed the expected pattern with EOP values of  $2 \times 10^{-5}$ , 0.75 and 0.8 obtained with the phages p2, 712, and p2.1, respectively<sup>20</sup>. The expected EOP value and the phage sensitivity pattern indicated that JH-80 was spontaneously expressing AbiV.

To verify that the Abi phenotype was indeed caused by expression of AbiV, we investigated the transcription of *abiV* in JH-80 using reverse transcriptase PCR (RT-PCR) as described previously<sup>20</sup>. The RT-PCR was performed on RNA isolated from JH-80, JH-20, JH-54, and JH-32 (Fig. 1). While levels of *abiV* transcription in JH-20 and JH-32 were highest, JH-80 indeed showed a higher transcription level compared to the wild type level in JH-54. A negative control was included by running an identical experiment just omitting the reverse transcriptase enzyme in the process, which demonstrated that the RNA was free of contaminating DNA (Fig. 1B). A positive control was performed using primers for the glycolytic genes *gapB* and *pfk* excluding artifacts due to RNA loss in the sample (data not shown). The above data demonstrates that *L. lactis* mutants can be isolated, which spontaneously express AbiV thereby conferring phage resistance to the cell. These findings are of potential value for the dairy industry since expression of AbiV was obtained without using genetic modification<sup>7,33,47</sup>. It was previously observed that the AbiV activity was not gene-dosage dependent against 936 phages when expressed from an expression vector or from a chromosomal insert<sup>20</sup>. However, the weak expression observed in the present study (Fig. 1A, lane 3) was not sufficient to confer resistance to 936 phages.

In an attempt to elucidate the mutation(s) in JH-80 leading to increased *abiV* transcription, we PCR-amplified a ca. 1300 bp region (nucleotides 1828 to 3140 on Genbank acc.nr: AF324839) of the chromosome upstream of *abiV*. The size of the DNA fragment was the same as a fragment amplified from wild type *L. lactis* MG1363, which indicates that no DNA deletions or rearrangements had occurred immediately upstream of *abiV* (results not shown). The PCR-amplified DNA fragment was sequenced and revealed three point mutations ca 400 bp upstream of *abiV* and further three point mutations about 1 kbp upstream of *abiV*. All mutations were located in an upstream gene *trans* encoding a putative transposase. This gene was previously demonstrated to be silent in *L. lactis* MG1363<sup>20</sup> and it is therefore not probable that the increased level of *abiV* transcription was caused by an altered protein structure of a *trans* gene product. We did not identify any terminator structures between the point mutations and *abiV*. On the other hand, the point

mutations did not reveal an obvious lactococcal consensus promoter sequence and we were thus unable to reveal the genetic determinant leading to increased transcription of *abiV* (data not shown).



**Fig 1.** Reverse transcriptase PCR (25 PCR cycles) carried out on isolated RNA. (A) Experiment done with reverse transcriptase enzyme, (B) Control without reverse transcriptase. Lanes 1-4 represents: JH-80 (spontaneous mutant), JH-20 (high expression of *AbiV*), JH-54 (wt), JH-32 (insertional mutant), respectively. L is Generuler 100-10,000 DNA ladder (Fermentas)

**Transfer of *abiV* by chromosomal conjugation.** Most known *Abi* mechanisms are plasmid encoded though it has been argued that this overrepresentation could be due to the technical advantages of isolating the plasmid encoded *Abi* systems<sup>5</sup>. *AbiV*, like a few other *Abi* mechanisms<sup>6,37,38</sup>, is located on the chromosome of its host (*L. lactis* MG1363) and is thus not readily transferable. Conjugation of chromosomal genetic material has been observed in *L. lactis*<sup>17,19</sup>, and a chromosomally encoded sex-factor was demonstrated to exist in *L. lactis* MG1363<sup>43,44</sup> thereby permitting lactococcal strains to exchange genetic material by chromosomal transfer and subsequent recombination. In the present study we took advantage of this ability, to transfer *abiV* from JH-32 (*Abi*<sup>+</sup>, *Erm*<sup>R</sup>, *FU*<sup>R</sup>)<sup>20</sup> to MG1614 (*Rif*<sup>R</sup>, *Strp*<sup>R</sup>)<sup>18</sup>. In short, donor and recipient were grown separately on plates and subsequently recovered with 0.9 % NaCl before they were mixed at ratios of 1:1, 1:3, and 1:9, plated (0.1 ml plate<sup>-1</sup>) on several plates, and then incubated anaerobically overnight at 36°C (growth at 36°C prevents excision of a pGhost9::*ISSI* insert in JH-32)<sup>20,28</sup>. The cells were then recovered in 0.9 % NaCl and incubated anaerobically (48 h, 30°C) on selective plates (erythromycin and rifampicin).

In the recently constructed strain JH-32, the insertional mutagenesis vector pGhost9::*ISSI*<sup>28</sup> was inserted immediately upstream of *abiV* on the bacterial chromosome and it was suggested that

the observed transcription of *abiV* was due to a read-through from the erythromycin resistance gene in the inserted plasmid <sup>20</sup>. In a chromosomal transfer it is therefore likely that both the *Erm<sup>R</sup>* gene and *abiV* will be transferred together due to their close location. This allowed us to select for erythromycin resistance (*Erm<sup>R</sup>*) instead of using phage resistance as a selection marker for the donor phenotype. Rifampicin resistance (*Rif<sup>R</sup>*) was used to select for the recipient cell phenotype and transconjugants were thus isolated using selection for *Erm<sup>R</sup>* and *Rif<sup>R</sup>*. Additional unique resistance markers in donor (fluorouracil, *FU<sup>R</sup>*) and recipient (streptomycin, *Str<sup>R</sup>*) allowed us to test the transconjugants for phenotypes against which they were not selected. Using this approach, we minimized the risk of isolating false positives due to spontaneous mutations causing the resistance phenotype. Thus, isolated transconjugants (*Erm<sup>R</sup>*, *Rif<sup>R</sup>*) could be tested for resistance to fluorouracil (*FU<sup>R</sup>*, donor) and streptomycin (*Str<sup>R</sup>*, recipient). Transconjugants with the phenotype *FU<sup>S</sup>*, *Erm<sup>R</sup>*, *phage<sup>R</sup>*, *Rif<sup>R</sup>*, *Str<sup>R</sup>* are expected to originate from MG1614 (*Rif<sup>R</sup>*, *Str<sup>R</sup>*) which have acquired *Erm<sup>R</sup>* and *phage<sup>R</sup>* from JH-32 by chromosomal transfer (Table 2). Seven *Erm<sup>R</sup>*, *Rif<sup>R</sup>* colonies were isolated after two days of anaerobic incubation (36°C). Five of these mutants were identified as donors with spontaneous *Rif<sup>R</sup>* mutations and one was identified as a recipient with a spontaneous *Erm<sup>R</sup>* mutation. However, one mutant (JH-83) had the recipient phenotype (*FU<sup>S</sup>* (grown on SA plates supplemented with 0.5 % glucose <sup>25</sup>), *Rif<sup>R</sup>* and *Str<sup>R</sup>*) in addition to being *Erm<sup>R</sup>* and resistant to phage p2 with the same EOP as JH-20 (EOP of ca 10<sup>-4</sup>). Spontaneous *Str<sup>R</sup>* mutations are very rare and since streptomycin was not used in the selection process, the subsequent test for *Str<sup>R</sup>* were used to determine the recipient origin of the mutant. The results therefore strongly indicate that chromosomal transfer of *Erm<sup>R</sup>* and *abiV* was responsible for the observed phenotype.

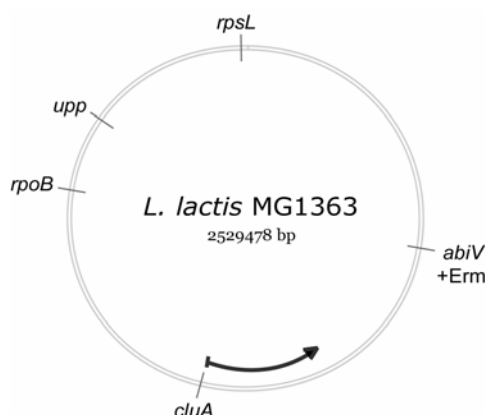
**TABLE 2.** Resistance phenotypes in conjugation experiments

	Phage	Erythromycin	Rifampicin	Streptomycin	Fluorouracil
JH-32 (D)	yes	yes	no	no	yes
MG1614 (R)	no	no	yes	yes	no
JH-83 (T)	yes	yes	yes	yes	no
Donor (D), recipient (R) and transconjugant (T). Selection for transconjugants done with erythromycin and rifampicin					

Theoretically, this phenotype could be explained both by transfer of genetic material from MG1614 to JH-32 and by transfer of genetic material from JH-32 to MG1614 followed by different subsequent recombination events. However, chromosomal gene transfer in *L. lactis* MG1363 occurs in the anti-clockwise direction <sup>17</sup> and the location of the resistance markers on the *L. lactis* MG1363 chromosome with, the *Erm<sup>R</sup>*/*phage<sup>R</sup>* markers being closest to the sexfactor, therefore makes the JH-

32 to MG1614 transfer most likely (Fig. 2). This is due to a gradual decrease in conjugation frequencies for anti-clockwise locations progressively more distant from the sex-factor.

PCR amplification of a chromosomal region upstream of *abiV* (including parts of the  $\text{Erm}^R$



**Fig. 2.** Genomic map of *L. lactis subsp. cremoris* MG1363 with indicated positions of genetic markers used in the conjugation experiments. *AbiV* is expressed from the erythromycin resistance ( $\text{Erm}$ ) promoter. Resistance to streptomycin, fluorouracil, and rifampicin is mediated by mutations in *rpsL*, *upp*, and *rpoB*, respectively. Chromosomal transfer is initiated at the sexfactor (including the *cluA* gene) and progresses in an anticlockwise direction (arrow)

gene in the JH-32 insertion) in JH-83 was performed. The patterns of amplified PCR fragments were identical in JH-32 and JH-83 while being different from the MG1614 pattern, which indicates that *abiV* and the  $\text{Erm}^R$  had been successfully transferred from *L. lactis* JH-32 to *L. lactis* MG1614 (data not shown).

The data indicates that *abiV* can be transferred between lactococcal strains by the means of chromosomal transfer. To our knowledge this is the first demonstration of a conjugal transfer of a chromosomally encoded phage resistance mechanism.

For industrial applications it is of great convenience to use phage resistance mechanisms encoded on conjugal plasmids, which is indeed the case for most of the isolated natural *Abi* and *R/M* mechanisms<sup>5,7</sup>. *AbiV* is flanked by two putative transposases (*lin* and *trans*) on the chromosome of *L. lactis* MG1363 (Gen.Bank acc.nr. AF324839)<sup>48</sup> and it is thus possible that *abiV* can be mobilized onto a conjugative plasmid due to the location among these mobile elements. However, more experiments are needed to test this hypothesis.

## ACKNOWLEDGMENTS

This work was funded in part by a graduate scholarship from the Technical University of Denmark to JH and a strategic grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada to SM.

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Submission number	1400000492	
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Applicant	DANMARKS TEKNISKE UNIVERSITET	
Number of applicants	5	
Country	DK	
Title	PHAGE RESISTANCE	
Documents submitted	eolf-pkda.xml pct101.GML eolf-requ.xml eolf-othd-000001.pdf (40 p.) eolf-appb-P000001.pdf (6 p.) eolf-othd-000002.txt Pct101.PDF (5 p.) referenc.inf	eolf-vlog.xml eolf-appb.xml eolf-fees.xml eolf-abst.txt eolf-seql.pdf (7 p.) pct101.1WO pct101u.gml
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Method of submission	Online	
Date and time receipt generated	02 July 2008, 15:06:46 (CEST)	



## PHAGE RESISTANCE

### Technical field of the invention

- 5 The present invention relates to the field of dairy science. In particular the present invention relates to methods for improving dairy starter culture quality.

### Background of the invention

- 10 The lactic acid bacterium *Lactococcus lactis* is used in milk fermentations world wide in the dairy industry to produce a variety of cultured dairy products. Phage infections can ruin the fermentation by inactivating the inoculated cultures. Phages are the major cause of fermentation failures during the manufacture of these cultured dairy products. There is thus a permanent need in the art for *L.*  
15 *lactis* starter cultures to perform at a high level of consistency and efficiency.

#### *Phages*

- Lactococcal phages are characterized by having relatively short latent periods and  
20 relatively large burst sizes. They are the major cause of fermentation failure leading to production loss in the dairy industry. Lactococcal phages are currently divided into eight distinct groups of which three groups namely "936", "c2" and "P335" are responsible for the vast majority of phage attacks in industrial fermentations. The genomes of the phages within one single group are highly  
25 conserved except for the P335 group.

- Industrial fermentations are carried out in large fermentation vats in a non-sterile environment. Prior to fermentation, the ingredients are usually pasteurized. However, the phages are often resistant to the pasteurization process. Presence of  
30 phages can lead to variations in flavor and texture of the fermented dairy product or even loss of the entire production with serious economical loss as a consequence. The dairy industry is therefore using a variety of methods in limiting

phage attacks. Such approaches include e.g. improved disinfection processes, rotation of starter cultures and application of phage resistant starter strains.

### *Phage defense mechanisms*

5

During evolution *L. lactis* has developed a series of defense mechanisms against phage attacks. These naturally occurring phage resistance mechanisms ( $\phi$ rm) has been studied extensively and also applied in industrial starter cultures. Most of the naturally occurring  $\phi$ rms are found on plasmids and they are classified into four

10 groups according to their mode of action: 1) adsorption inhibition, 2) blocking of phage DNA injection, 3) restriction/modification systems (R/M) and 4) abortive infection mechanisms (Abi). Among these defense mechanisms, the Abi systems are considered to be the most powerful due to their diverse mode of action and efficiency against the most common phages.

15

### *Abi mechanisms*

Abi mechanisms function in the phage life cycle subsequent to the injection of phage DNA into the bacterial cell - typically after expression of early phage genes.

20 As a consequence, the phage lytic cycle is terminated and usually the host dies. Very few viable phage progeny are thus released and the phenotypic outcome is a reduction in the number and size of plaques and thus a reduction of the severity of the phage infection.

25 To date, twenty-two lactococcal Abi systems have been isolated. These Abi systems target one, two or all three groups of the common phage species 936, c2, P335 with varying efficiency (EOP values from  $10^{-1}$  to  $<10^{-8}$ ) (fig 1).

Most of the isolated Abi systems are found on plasmids of which many are

30 conjugative. By sharing the  $\phi$ rms within the bacterial population, conjugation thus provides an adaptation strategy to the phage containing dairy environment. Only a few abi mechanisms have been isolated from the chromosome of *L. lactis*. This may partly be due to the fact that it is generally easier to isolate genes present on plasmids compared to isolation of genes present on chromosomes. The procedure

used in the present invention to isolate a  $\phi$ rm from the chromosome of *L. lactis* can be used to identify other  $\phi$ rms on the bacterial chromosome.

By isolating spontaneous phage resistant mutants with a similar phenotype with  
5 regards to efficiency against a range of phage species it is probably possible to identify strains expressing the abi without having to use genetic modification. Using this method, non-GMO phage resistant strains can thus be isolated. Use of non-GMO starter cultures may be an advantage in some case, in particular in relation to the fact that the legislation in some countries does not allow use of  
10 GMO. Furthermore, some consumers tend to prefer non-GMO derived products.

The point of interference with the phage life cycle has been determined to some degree for most of the Abi mechanisms:

- AbiA, AbiF, AbiK, AbiP, AbiR, and AbiT apparently interfere with phage DNA  
15 replication.
- AbiC apparently interfere with capsid production.
- AbiE, AbiI, and AbiQ apparently interfere with phage packaging.
- AbiB is apparently an RNase.
- AbiD1 seems to interfere with a phage RuvC-like endonuclease.
- 20 • AbiU apparently delays phage transcription.
- AbiZ apparently causes premature lysis of the infected cell.

These very diverse modes of action are most likely the reason for the very low degree of protein homology that exists between the different Abi mechanisms.

25 Though the point of action in the phage life cycle has been determined, the phage protein interacting with the Abi mechanism has only been identified in AbiA, AbiD1, AbiK and AbiP. An increasing number of phage genomes are being sequenced providing a bulk of sequence data in which numerous putative proteins  
30 are found. However, experimental evidence for the function of these proteins are lacking behind.

Several phage resistant strains of *L. lactis* have been constructed by introducing abi systems in phage sensitive industrial starter cultures. However, extensive use

of these bacterial cultures leads to problems with emergence of phage mutants capable of overcoming the introduced abi systems.

The evolutionary "arms race" between phage mutants and bacterial  $\phi$ rms means  
5 that there is a constant need in the art for identifying novel natural  $\phi$ rms. There is a particular need in the art for finding novel Abi-mechanisms that interact with previously unknown targets in the phage. Furthermore there is a need in the art for novel Abi-mechanisms in *Lactococcus* bacteria that do not classify as GMO. Finally there is a need in the art for identifying  $\phi$ rms that provide efficient  
10 protection against phages.

### Summary of the invention

The present invention thus relates to a polynucleotide conferring at least one  
15 phage resistance mechanism to a *Lactococcus* bacterium, wherein said polynucleotide encodes a polypeptide according to SEQ ID NO 1 and/or SEQ ID NO 2, and/or SEQ ID NO7 or a fragment or variant thereof. The present invention furthermore relates to the polypeptides, uses thereof, expression vectors and cells expressing these polypeptide sequences. The present invention also relates to  
20 methods for producing fermented dairy products as well as the products resulting from these processes.

The present invention finally relates to methods for identifying  $\phi$ rms on  
25 chromosomal DNA.

The novel  $\phi$ rm(-s) according to the present invention provide a number of advantages as described in the following.

### Brief description of the figures

30 Figure 1: Characteristics of the twenty-two (including AbiV from the present invention) Abi mechanisms isolated to date.



Figure 2: The sequence from GenBank (acc.nr AF324839) containing orf1 which surprisingly turned out to have the capability to function as a  $\phi$ rm according to the present invention. Fig 2A: the strains with the transposon containing vector pGhost9::ISS1 inserted on the chromosome. Arrows indicate the position and direction of the inserted ISS1 sequences. The presence of a promoter and the  $\phi$ rm<sup>+</sup> phenotype is indicated to the right. Fig 2B: the strains with the cloned fragment including orf1. The lines represent the cloned DNA fragment, and the x in JH-24 represent the position of the frame shift mutation introduced into this strain.

10

Figure 3: Time course experiment of a phage infection. Samples are taken during infection of phage resistant *Lactococcus lactis* strain JH-20 (upper panel) and phage sensitive *Lactococcus lactis* strain JH-16 (lower panel) with p2 phage. The experiment was run for 120 min and samples were taken at: -10, 0, 10, 20, 30, 40, 50, 60, 90 and 120 minutes. Total DNA was isolated from the cells and restricted with *EcoRI*. The resulting restriction fragments are representing *EcoRI* digested p2 DNA. Band 1.3 kb and 4 kb are spanning the *cos* site which marks the extremities of the phage DNA. The *cos* site is cut during packaging of phage DNA in the lytic life cycle of the wt phage, revealing mature phage DNA molecules in units of one genome. In the phage resistant Abi mutant, the *cos* site is not cut resulting in non-mature phage DNA that can not be packed into the phage capsids. The figure thus shows that production of mature phage DNA is significantly decreased in the strains containing the AbiV mechanism.

Figure 4: DNA sequence of the 1.3 kb DNA fragment (bp 1021-2320 in GenBank acc.nr AF324839) cloned in vector pJH2. This fragment comprises orf1 (bp 1276-1878) encoding the  $\phi$ rm. Ribosome binding site is underlined in nucleotides matching the lactococcal consensus sequence (AGAAAGGAGGT). The translated amino acids are shown below the DNA sequence.

30

Figure 5: DNA sequence of the 499 bp DNA fragment from phage p2 containing orf26 and the upstream region towards orf27. Ribosome binding site is underlined in nucleotides matching the lactococcal consensus sequence (AGAAAGGAGGT). The translated amino acids are shown below the DNA sequence.

35

Figure 6: Reverse transcriptase PCR carried out on isolated RNA. (A) Experiment done with reverse transcriptase enzyme. (B) Control without reverse transcriptase. Lanes 1-4 represents: JH-80 (spontaneous mutant), JH-20 (high expression of AbiV), JH-54 (wt), JH-32 (insertional mutant), respectively. L is 5 Generuler ladder (Fermentas). The present invention will now be described in more detail in the following.

## **Detailed description of the invention**

### 10 Definitions

Prior to discussing the present invention in further details, the following terms and conventions will first be defined:

- 15 Phages: A bacteriophage (from 'bacteria' and Greek phagein, 'to eat') is any one of a number of virus-like agents that infect bacteria. The term is commonly used in its shortened form, phage. Typically, bacteriophages consist of an outer protein shell (called capsid or head) enclosing genetic material. The genetic material can be ssRNA, dsRNA, ssDNA, or dsDNA between 5 and 500 kilo base pairs long with
- 20 either circular or linear arrangement. Bacteriophages are much smaller than the bacteria they destroy - usually between 20 and 200 nm in size. Phages according to the present invention have the ability to infect bacteria of the genus *Lactococcus*.
- 25 Phage resistance mechanism: A functional phage resistance mechanism is herein meant to be a mechanism that directly inhibits the phage lytic life cycle. However, phage resistance mechanisms as used herein furthermore denote mechanisms that works in synergy with a phage encoded product. As an example hereof, the present invention relates to use of SEQ ID NO 1 for conferring phage resistance to
- 30 bacterial cells as well as the use of SEQ ID NO 1 in combination with SEQ ID NO 2 for obtaining an even more efficient phage resistance mechanism than was possible when only using SEQ ID NO 1.

*Lactococcus*: is a lactic acid bacterial genus of five major species formerly included as members of the genus *Streptococcus* Group N and related species. They are gram-positive bacteria, and they are typically spherical or ovoid, 0.5–1.2 µm by 0.5–1.5 µm, and occur in pairs and short chains. They are non-spore forming and are not motile. The type species for the genus is *L. lactis* which in addition have two subspecies *lactis* and *cremoris*. *Lactococcus* is commonly used in the dairy industry in the manufacture of fermented dairy products. They can be used in single strain starter cultures, or in mixed strain cultures comprising other strains of *Lactococcus* or lactic acid bacteria such as e.g. *Leuconostoc*,  
 5  
 10 *Lactobacillus* and *Streptococcus*.

A fragment: A fragment according to the present invention is herein defined as a fragment of a polypeptide being at least 100 amino acids, preferably at least 110, more preferably at least 120 amino acids. With regards to SEQ ID NO 1, the  
 15 fragment is preferably at least 100 amino acids in length, more preferably at least 125 amino acids in length, more preferably at least 150 amino acids in length, more preferably at least 175 and most preferably at least 190 amino acids in length.

Promoter: The term "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. As used herein the term promoter shall include any portion of genomic DNA (including genomic DNA disclosed herein), which is capable of initiating  
 25 expression of but not limited to operably linked nucleotide sequences at levels detectable above background. In the context with the present invention a "strong promoter" shall be understood as a promoter which results in expression of a polypeptide according to the invention, wherein the level of expression is significantly higher compared to the endogenous homologous promoter in the  
 30 *Lactococcus* genome. The level of expression can be detected and/or measured by e.g. Northern blot, real-time PCR, reporter gene assays, etc.

Expression vector: A vector is a component or composition for facilitating cell transduction or transfection by a selected nucleic acid, or expression of the nucleic  
 35 acid in the cell. Vectors include, e.g., plasmids, cosmids, viruses, BACs, PACs, P1,

YACs, bacteria, poly-lysine, as well as linear nucleotide fragments etc. An "expression vector" is a nucleic acid construct or sequence, generated recombinantly or synthetically, with a series of specific nucleic acid elements that permit transcription of a particular nucleic acid sequence in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. The expression vector typically includes a nucleic acid to be transcribed operably linked to a promoter. The nucleic acid to be transcribed is typically under the direction or control of the promoter. The expression vector may replicate autonomously in the host cell or may integrate into the host genome after the transfection or transduction and replicate as part of the genome. Finally "an expression vector" encoding more than one polypeptide sequences according to the present invention comprises the situation wherein one expression vector comprises polynucleotide sequences encoding more than one polypeptide product as well as the situation wherein the polynucleotide sequences are cloned into two different expression vectors.

pGhost9::ISS1: The term "pGhost9::ISS1" covers a vector with an antibiotic resistance marker, a *Lactococcus* replicon, and preferably also an *E. coli* replicon. The replicon is thermosensitive allowing for selection for integration into the host chromosome. Also the vector contains an insertion sequence that enables random integration of the vector into the host chromosome. It follows that vectors with similar functions may be used in connection with the present invention.

Identity: The term "identity" or "sequence identity" is a measure of the degree of identity between polynucleotide sequences on a nucleotide-by-nucleotide basis or amino acid-by-amino acid basis, respectively over a window of comparison. Sequences according to the present invention have an identity of at least 70% to SEQ ID NO 1, or a fragment thereof.

Food products: Food products according to the present invention include milk based products that have been subject to fermentation processes. Examples thereof include: sour cream, crème fraîche, buttermilk, butter, cheese, cottage cheese, quark, cream cheese, fromage frais, yoghurt, etc. However, other types of food products may also be produced using fermentation or fermentative

microorganisms according to the present invention such as e.g fruit juices, fermented vegetables/fruits, processed meat products, etc.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention.

In a first aspect the present invention thus relates to an isolated polynucleotide sequence that encodes a polypeptide with at least 70% identity, preferably at least 75% identity, more preferably at least 80% identity, more preferably at least 85% identity, more preferably at least 70% identity, preferably at least 75% identity, more preferably at least 80% identity, more preferably at least 85% identity, more preferably at least 90% identity, and most preferably at least 95% identity with SEQ ID NO 1 (AbiV from *Lactococcus lactis*), or a fragment thereof, and wherein expression of said polynucleotide confers at least one phage resistance mechanism to a *Lactococcus* bacterium. This polynucleotide sequence is found naturally in the *Lactococcus* bacterium, but it is normally not transcriptionally active. It has surprisingly been found that expression of this polypeptide confers a previously unknown phage resistance mechanism to the bacterium.

A second aspect of the present invention relates to an isolated polynucleotide derived from a *Lactococcus lactis* phage that encodes a polypeptide with at least 70% identity, preferably at least 75% identity, more preferably at least 80% identity, more preferably at least 85% identity, more preferably at least 95% identity, preferably at least 97% identity, and most preferably at least 99% identity with SEQ ID NO 2, or a fragment thereof and/or an isolated polynucleotide that encodes a polypeptide with at least 70% identity, preferably at least 75% identity, more preferably at least 80% identity, more preferably at least 85% identity, more preferably at least 95% identity, preferably at least 97% identity, and most preferably at least 99% identity with SEQ ID NO 7. Optionally, the polynucleotide sequence may encode at least one of SEQ ID NO 1, SEQ ID NO 2, and SEQ ID NO 7 or any variant thereof in the form of one or more polynucleotide sequences.

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SEQ ID NO 2 and SEQ ID NO 7 are phage proteins. The inventors have found out that these proteins most likely need to be mutated in order for the phage to escape the phage resistance mechanism conferred by expression of SEQ ID NO 1 or variants thereof. Phage proteins according to the present invention therefore  
5 have at least 70% identity with SEQ ID NO 2 and/or SEQ ID NO 7 in order to provide functional phage protein that may suppress the effects of emergence of mutated phage protein that could potentially suppress the effects of the translated SEQ ID NO 1 protein or variants thereof. In a preferred embodiment according to the present invention, polynucleotide sequences encoding both SEQ ID NO 1 or  
10 variants thereof as well as SEQ ID NO 2 and/or SEQ ID NO 7 or variants thereof, are thus provided thus conferring highly efficient phage protection mechanisms to a host cell. It furthermore follows that the invention relates to expression vectors as well as *Lactococcus* bacteria and/or starter cultures comprising polynucleotide sequences encoding such polypeptide sequences.

15

In a third aspect, the present invention relates to an isolated polypeptide conferring at least one phage resistance mechanism to a *Lactococcus* bacterium, wherein said polypeptide is selected from one or more of the group consisting of:  
a polypeptide with at least 70% identity with SEQ ID NO 1, or a fragment thereof,  
20 a polypeptide with at least 70% identity with SEQ ID NO 2, or a fragment thereof, and a polypeptide with at least 70% identity with SEQ ID NO 7, or a fragment thereof.

A fourth aspect relates to the use of one or more polynucleotides according to the  
25 present invention and/or one or more polypeptides according to the present invention for improving phage resistance in a *Lactococcus* bacterium.

A fifth aspect relates to a method for fermenting food product, said method comprising the step of adding one or more of the components according to the  
30 present invention. The invention furthermore relates to products that can be obtained and/or are obtained using this method.

A sixth aspect relates to a method for obtaining phage resistant bacterial cells, said method comprising use of pGhost9::ISS1 (or similar systems) for random  
35 insertion into a bacterial cell and subsequently screening and selecting for phage

resistant cells. The invention furthermore relates to cells that can be obtained and/or are obtained by such methods. In a preferred embodiment, the cell is a *Lactococcus* bacterium wherein a polynucleotide encoding SEQ ID NO 1 (or a variant thereof) is transcriptionally active.

5

A final aspect relates to a *Lactococcus* bacterium that expresses at least one polypeptide selected from the group consisting of: a polypeptide with at least 70% identity with SEQ ID NO 1, or a fragment thereof, a polypeptide with at least 70% identity with SEQ ID NO 2, or a fragment thereof, and a polypeptide with at least  
10 70% identity with SEQ ID NO 7, or a fragment thereof.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

15 The invention will now be described in further details in the following non-limiting examples.

## EXAMPLES

### 20 **Example 1**

#### *Bacterial strains, plasmids, and media*

Strains and plasmids used in this invention are listed in table 1. *Escherichia coli* was grown at 37°C in LB medium. *Lactococcus lactis* was grown in M17 with the  
25 supplement of 0.5% glucose (GM17). Lactococci were grown at 30°C except strains containing the thermo sensitive vector pGhost9::ISS1. These strains were grown at 28°C for replication of the vector or 36°C to avoid replication. When appropriate, antibiotics were added as follows: *E. coli*, 100 µg/ml of ampicillin, 10 µg/ml of chloramphenicol, 150 µg/ml of erythromycin; for *L. lactis*, 5 µg/ml of  
30 chloramphenicol, 3 µg/ml of erythromycin.

Table 1

**List of bacteria, phages and plasmids used in the invention**

Bacterial strain, phage or plasmid	Characteristic	Source
<b><i>Lactococcus lactis</i></b>		
SMQ-86	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> . Multiple plasmids, pSA3, host for the tested P335 phages. Erm <sup>R</sup>	(2)
IL1403	<i>Lactococcus lactis</i> subsp. <i>lactis</i> IL1403, host for some 936 phages	(1)
MB112	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363, $\Delta upp$ , Host for 936 and c2 phages	(7)
JH-20	MB112 (pJH2); Cam <sup>R</sup> , Abi <sup>+</sup>	This Invention
JH-22	IL1403 (pJH2); Cam <sup>R</sup> , Abi <sup>+</sup>	This Invention
JH-23	SMQ-86 (pJH2); Cam <sup>R</sup> , Abi <sup>+</sup>	This Invention
JH-24	MB112 (pJH3); Cam <sup>R</sup> , Abi <sup>-</sup>	This Invention
JH-25	MB112 (pJH4); Cam <sup>R</sup> , Abi <sup>+</sup>	This Invention
JH-26	MB112 (pJH5); Cam <sup>R</sup> , Abi <sup>+</sup>	This Invention
JH-32	MB112 (pGhost9::ISS1 inserted in Lin at bp1962); Erm <sup>R</sup> , grown at 36° C, Abi <sup>+</sup>	This Invention
JH-46	MB112 (pGhost9::ISS1 inserted in Lin at bp1962); Erm <sup>R</sup> , grown at 36° C, Abi <sup>+</sup>	This Invention
JH-47	MB112 (pGhost9::ISS1 inserted in Lin at bp2296); Erm <sup>R</sup> , grown at 36° C, Abi <sup>+</sup>	This Invention
JH-48	MB112 (pGhost9::ISS1 inserted in Lin at bp2240); Erm <sup>R</sup> , grown at 36° C, Abi <sup>+</sup>	This Invention
JH-49	JH-32 cured for pGhost9, leaving ISS1 in place; Abi <sup>-</sup>	This Invention
JH-50	JH-46 cured for pGhost9, leaving ISS1 in place; Abi <sup>-</sup>	This Invention
JH-51	JH-47 cured for pGhost9, leaving ISS1 in place; Abi <sup>-</sup>	This Invention
JH-52	JH-48 cured for pGhost9, leaving ISS1 in place; Abi <sup>-</sup>	This Invention
JH-53	MB112 (pJH6); Cam <sup>R</sup> , Abi <sup>-</sup>	This Invention
JH-54	MB112 (pLC5); Cam <sup>R</sup> , Abi <sup>-</sup>	This Invention
JH-80	MB112 (spontaneous mutation to express $\phi rm$ ); Abi <sup>+</sup>	This Invention



Bacterial strain, phage or plasmid	Characteristic	Source
JH-81	transconjugant with transferred $\phi$ rm and $Erm^R$ from JH-32 to LKH208; $Abi^{+}$ , $Erm^R$ , $Rif^R$ , $Strep^R$ , $FU^S$	This Invention
MG1614	MG 1363 $Rif^R$ , $Strep^R$ (M.Gasson)	(5)
<b><i>Escherichia coli</i></b>		
EC1000	RepA <sup>+</sup> MC1000, Km <sup>R</sup>	(4)
JH-56	EC1000 (pJH7), $Erm^R$	This Invention
JH-57	EC1000 (pJH8), $Erm^R$	This Invention
JH-58	EC1000 (pJH9), $Erm^R$	This Invention
JH-59	EC1000 (pJH10), $Erm^R$	This Invention
TOP10F'	Chemically competent cells from the TOPO TA cloning kit	Invitrogen
JH-19d	EC1000 (pJH6), Cam <sup>R</sup>	This Invention
<b>phages</b>		
p2	Small isometric headed, 936 species	S.M
sk1	Small isometric headed, 936 species	F.V
jj50	Small isometric headed, 936 species	F.V
712	Small isometric headed, 936 species	S.M
P008	Small isometric headed, 936 species	S.M
bIL170	Small isometric headed, 936 species	S.M
c2	Prolate headed, c2 species	S.M
bIL67	Prolate headed, c2 species	S.M
ml3	Prolate headed, c2 species	S.M
eb1	Prolate headed, c2 species	S.M
ul36	Small isometric headed, P335 species	S.M

Bacterial strain, phage or plasmid	Characteristic	Source
KITI	Small isometric headed, P335 species	S.M
Ø31	Small isometric headed, P335 species	S.M
Ø50	Small isometric headed, P335 species	S.M
Q33	Small isometric headed, P335 species	S.M
Q30	Small isometric headed, P335 species	S.M
P335	Small isometric headed, P335 species	S.M
p2.1	Small isometric headed, 936 species, deletion in <i>orf26</i>	This Invention
sk1.1	Small isometric headed, 936 species, nonsense mutation in <i>orf26</i>	This Invention
jj50.1	Small isometric headed, 936 species, nonsense mutation in <i>orf25</i> (homologue to p2 <i>orf26</i> )	This Invention
P008.1	Small isometric headed, 936 species, nonsense mutation in <i>orf33</i> (homologue to p2 <i>orf26</i> )	This Invention
bIL170.1	Small isometric headed, 936 species, nonsense mutation in <i>e24</i> (homologue to p2 <i>orf26</i> )	This Invention
c2.1	Prolate headed, c2 species, mutation in <i>e11</i> (homologue to p2 <i>orf26</i> )	This Invention
<b>plasmids</b>		
pCI372	Shuttle vector for <i>E.coli</i> and <i>L.lactis</i> . No promoter in front of multiple cloning site; Cam <sup>R</sup>	(3)
pLC5	Expression vector for <i>L. lactis</i> and <i>E.coli</i> . Promoter in front of <i>PstI</i> site used for cloning; Cam <sup>R</sup>	This Invention
pGhost9::ISS1	pGhost9::ISS1, temperature sensitive vector with insertion sequence used for random mutagenesis, Erm <sup>R</sup>	(6)
pJH2	bp 1021-2320 on Lin sequence* cloned in <i>PstI</i> site of pLC5; Cam <sup>R</sup>	This Invention
pJH3	pJH2, restricted in <i>ClaI</i> site of AbiV and filled with Klenow, gives frameshift mutation; Cam <sup>R</sup>	This Invention
pJH4	pJH2 isolated from JH22	This Invention
pJH5	pJH2 isolated from JH23	This Invention
pJH6	bp 1021-2320 on Lin sequence* cloned in <i>PstI</i> and <i>XbaI</i> sites of pCI372	This Invention
pJH7	<i>HindIII</i> rescue of pGhost9::ISS1 with flanking chromosomal DNA from JH-32	This Invention

Bacterial strain, phage or plasmid	Characteristic	Source
pJH8	<i>HindIII</i> rescue of pGhost9::ISS1 with flanking chromosomal DNA from JH-46	This Invention
pJH9	<i>HindIII</i> rescue of pGhost9::ISS1 with flanking chromosomal DNA from JH-47	This Invention
Lin sequence refers to GeneBank acc nr AF324839		
Cam <sup>R</sup> , chloramphenicol resistance; Amp <sup>R</sup> , ampicillin resistance; Erm <sup>R</sup> , Erythromycin resistance; Km <sup>R</sup> , Kanamycin resistance; Rif <sup>R</sup> , Rifampicin resistance; Strep <sup>R</sup> , Streptomycin resistance; FU <sup>R</sup> fluorouracil resistance		
Abi <sup>+</sup> , phage resistance phenotype; Abi <sup>-</sup> , phage sensitive phenotype		
F.V = Finn K. Vogensen, University of Copenhagen		
S.M = Sylvain Moineau		

**Example 2***Bacteriophage propagation and assays.*

Bacteriophages used in this invention are listed in table 1. Bacteriophages sk1 and  
5 jj50 were kindly provided by F.K.Vogensen (University of Copenhagen). Prior to  
use all phages were purified two times by picking a single plaque with a sterile  
Pasteur pipette and plating it on a sensitive host. Propagation of phages to obtain  
high titer lysates was performed in two steps:

10 In the first propagation a single plaque was transferred into a fresh ON culture of  
a sensitive host inoculated (1%) in GM17 supplemented with 10 mM  $\text{CaCl}_2$  and  
incubated at 30°C (or 36°C in the case of pGhost9::ISS1 containing host strains)  
until lysis. The lysate was filtered through a 0.45  $\mu\text{m}$  syringe filter.

15 The second propagation was performed by inoculating an exponentially growing  
host culture at  $\text{OD}_{600} = 0.2$  with phages from the first propagation ( $10^4$  pfu/ml) in  
GM17 + 10 mM  $\text{CaCl}_2$ .

The culture was then incubated with agitation (200rpm) until lysis at the same  
20 temperature as for the first propagation. The lysate was filtered (0.45  $\mu\text{m}$  filter).  
The titer of phage lysates was determined using conventional methods.

Efficiency of plaquing (EOP) was calculated by dividing the titer on the tested  
strain with the titer on the sensitive wt strain. Adsorption assays were conducted  
25 as described by Sanders and Klaenhammer(17) except a 5 min incubation period  
was used instead of 15 min. Cell survival was assayed by the method of Behnke  
and Malke (2) using a multiplicity of infection (MOI) of 5. One-step growth assay  
(and determination of burst size) and center of infection (COI) assay was  
performed as described previously (14) by using MOIs of 0.2 and 0.5,  
30 respectively. ECOI (efficiency of COI) was determined by dividing the number of  
COI from the resistant strain by the number of COI from the sensitive strain.  
Replication of phage DNA was followed in a time course experiment using the  
method of Hill *et al.* (8). Visualization of phage DNA by labeling with the  
fluorescent dye SYBR-Gold was performed as described by Noble and Fuhrman

(15) with the following modifications: The original SYBR-Gold solution was diluted ( $\times 1000$ ). Phage lysate to be stained was treated with 1  $\mu\text{g/ml}$  DNase and RNase and incubated for 30 min at 37°C. The lysate was stained with the diluted SYBR-Gold to give 2.5% final concentration (vol/vol) diluted SYBR-Gold and left ON at 4  
 5 °C in the dark. One  $\mu\text{l}$  of the labeled phage stock was mixed with 1  $\mu\text{l}$  exponentially growing cell culture and visualized under a Zeiss axioplan epifluorescence microscope.

### Example 3

#### 10 *Mutagenesis with pGhost9::ISS1*

Random integration of the vector pGhost9::ISS1 into the chromosome of MB112 and subsequent cloning of flanking chromosomal DNA was performed essentially using the method of Maguin *et al.* (10). The method of Maguin, however, is  
 15 normally used to identify inactivation of genes by randomly inserting the construct in chromosomal genes, thereby inactivating them. Subsequent selection for a desired phenotype enables screening for strains containing a loss of function mutation. The fact that all inspected mutants in the present invention had insertions in non coding regions or genes upstream of orf1 together with the  
 20 observation that presence of the complete vector pGhost9::ISS1 was needed for the  $\text{Abi}^+$  phenotype led to the hypothesis that the *abiV* gene (orf1) was transcribed from the promoter encoding the erythromycin resistance gene in pGhost9::ISS1 (fig 2). Previous studies have reported promoter activity in the ISS1 sequence (5). No effect on phage resistance phenotype of such promoter  
 25 activity was observed in the present invention. It has not previously been shown that random insertion of the vector and subsequent transcription from the promoter of the erythromycin resistance gene can be used to activate existing biological mechanisms, such as e.g. *Abi*-mechanisms.

30 To ensure that the mutations in the isolated strains had arisen from independent events, the integration step (growth at 37°C) was performed on 12 separate cultures. After the integration step, the cultures were diluted  $\times 10.000$  in GM17 + 3  $\mu\text{g/ml}$  Erythromycin and left for phenotypic expression ON at 37°C. These cultures were inoculated (1%) and when growing exponentially aliquots were

removed. 10 mM CaCl<sub>2</sub> (final concentration) was added to these aliquots before inoculating with phage sk1 (MOI > 1). After 10 min incubation at 37°C the cultures were spread on selective GM17+Erm plates. A number of phage resistant colonies were isolated and purified from each of the 12 independent cultures. Four strains were chosen from independent cultures to identify the location of the inserted pGhost9::ISS1. This was performed by rescuing of the inserted vector and cloning of flanking chromosomal DNA. The cloned chromosomal DNA fragments were subsequently sequenced

#### 10 **Example 4**

##### *DNA isolation and manipulation*

Plasmid DNA was isolated from *E. coli* and *L. lactis* using the QIAprep Spin Miniprep Kit (Qiagen); for *L. lactis* however, lysozyme (15 mg/ml) was added to buffer p1 and the solution with the resuspended cells was incubated at 37°C for 30 min before proceeding with the manufacturers protocol. Phage DNA was prepared using the Qiagen Lambda Maxi Kit (Qiagen) with the addition of proteinase K (20mg/ml) to buffer L3 and subsequent incubation at 65°C for 30 min before adding buffer L4. Total intracellular DNA was isolated using the method of Hill *et al.* (8). Restriction enzymes, T4 DNA ligase and Klenow fragment (Fermentas) were used according to the manufacturer's instructions. Electroporation of *E. coli* and *L. lactis* was performed as described previously (13). The DNA fragment corresponding to bp 1021 to 2320 (fig. 4) in the GenBank sequence AF324839 was subcloned in the TOPO TA cloning kit prior to cloning in pCI372 and pLC5.

#### **Example 5**

##### *DNA sequencing and sequence analysis of DNA and protein*

Oligonucleotide sequences used for plasmid constructions and sequencing: For sequencing the flanking chromosomal DNA of the rescued pGhost9::ISS1 inserts a primer located in the ISS1 was used (5'-GAAGAAATGGAACGCTC-3').

Phage genome sequencing was performed with an ABI prism 3700 apparatus from the genomic platform at the research center of the Centre Hospitalier de l'Université Laval using a set of oligonucleotides previously used for sequencing of 936 phage genomes (11).

5

Sequence data was assembled using the Staden Pregap4 version 1.5. Sequence homology searches in databases were done using BLAST (1). Molecular weight and pI of the investigated proteins were estimated using the Protein Calculator at the website: <http://www.scripps.edu/~cdputnam/protcalc.html>

10

### Example 6

*A phage resistance mechanism ( $\varphi$ rm) is found on the chromosome of Lactococcus lactis subsp.cremoris MG1363*

15 *L. lactis* subsp. *cremoris* MG1363 is sensitive to infection of phages from the 936 and c2 species. In this invention a transposon mutagenesis system (described in details in (10)) was used to identify a novel  $\varphi$ rm on the chromosome of MG1363. The system (pGhost::ISS1) comprises the vector pGhost9 containing an erythromycin resistance gene ( $Em^r$ ) and the ISS1 insertion sequence which allows  
20 for random integration of the construct into the host chromosome. Due to a thermosensitive origin of replication (plasmid is not replicating at 37°C) it is possible to select for mutants with the construct inserted in the chromosome by growing at 37°C in the presence of erythromycin, allowing for phenotypic expression by growing at selective conditions ON.

25

This selection was done for a number of independently grown cultures resulting in isolation of independent integration events. These cultures were screened for resistance to phage sk1 by selecting colonies growing on erythromycin plates in the presence of phages (MOI>1). The frequency of mutations conferring phage  
30 resistance was 100 times higher in cultures with mutants containing the pGhost::ISS1 inserts compared to the control cultures in which the phage resistance was caused by spontaneous mutations. This clearly indicates that the mutations in the phage resistant mutants containing pGhost9::ISS1 in most cases were caused by the insertion of this construct.

A number of  $Em^r/\phi rm^+$  colonies were isolated. From four of these independently mutagenized cultures, the inserted construct was obtained along with a piece of flanking chromosomal DNA. Sequence analysis revealed insertions on the chromosome corresponding to bp 1962 (strains JH-32 and JH-46), bp 2240 (JH-48) and bp 2296 (JH-47) on the sequence available in GenBank under the accession number AF324839 (hereafter designated Lin). Bp 1021 to bp2320 therein corresponds to SEQ ID NO 6. All strains had insertions in the intergenic region between two genes (designated *orf1* and *trans*) or in the 3' end of the trans gene (fig 2A). There are no genes in the same orientation immediately downstream of *orf1* and since the mutagenizing constructs were all inserted in the same orientation pointing towards *orf1* it was hypothesized that *orf1* is encoding a  $\phi rm$  which is transcribed from the  $Em^r$  gene promoter when pGhost::ISS1 is inserted upstream of *orf1*. Curing the strains for the vector (leaving a single copy of ISS1 at the insertion site) resulted in  $\phi rm^-$  phenotype supporting the hypothesis that a promoter in pGhost::ISS1 is needed for transcription of *orf1* and the resulting  $\phi rm^+$  phenotype. This implies that *orf1* is silent in wt MG1363 which is supported by the phage sensitive phenotype of this strain.

### Example 7

#### 20 Identification of *orf1* as a $\phi rm$

To test if *orf1* is a  $\phi rm$ , a fragment corresponding to bp 1021 to 2320 on the Lin sequence was cloned in the shuttle vector pCI372 (pJH6) and in the expression vector pLC5 (pJH2). These constructs were transformed in MB112 and the resulting strains (JH-53 and JH-20, respectively) were tested by cross streaking assay for resistance to phage p2. JH-53 containing pJH6 with no promoter upstream of *orf1* showed no phage resistance phenotype. In comparison, JH-20 containing pJH2 with *orf1* cloned downstream of a strong promoter revealed phage resistance phenotype.

30

To verify *orf1* as being the  $\phi rm$ , a frameshift mutation was introduced in *orf1* by filling a unique *ClaI* site with Klenow fragment followed by ligation and transformation of this vector (pJH3) in wt MB112. The mutated *orf1* was sequenced verifying the frameshift mutation. The resulting strain JH-24 had a



phage sensitive phenotype and it was therefore concluded that *orf1* is encoding a  $\phi$ rm.

**Example 8**

5 *The isolated  $\phi$ rm is effective against phages of the 936 and c2 species*

The three phage species 936, c2 and P335, known to be responsible for the majority of phage caused fermentation failures were tested for their sensitivity to the  $\phi$ rm. Four strains of the 936 species were tested against JH-20. Efficiency of  
10 plaquing (EOP) values around  $10^{-4}$  were obtained for phages p2, sk1 and jj50 while phage 712 was insensitive to the  $\phi$ rm (Table 2). pJH2 was inserted into the host JH-22 (*L. lactis* subsp. *lactis* IL1403) which is sensitive to the 936 phages P008 and bIL170. When tested against these phages the  $\phi$ rm revealed EOP values around  $10^{-4}$ . Similar values were obtained when testing JH-20 against four phages  
15 of the c2 species (Table 2). Similar EOP values were obtained for MB112 and JH-54 when tested against the 936 and c2 phage species (data not shown), thus ruling out the possibility for the vector pLC5 being responsible for the  $\phi$ rm<sup>+</sup> phenotype.

20 Table 2

	Phage	Host strain	EOP
936 species <sup>a</sup>	sk1	JH-20	$2.7 \pm 1.4 \times 10^{-4}$
	p2	JH-20	$4.8 \pm 1.8 \times 10^{-4}$
	jj50	JH-20	$8.3 \pm 0.5 \times 10^{-5}$
	712	JH-20	$1.1 \pm 0.2$
	P008	JH-22	$3.8 \pm 1.5 \times 10^{-4}$
	bIL170	JH-22	$3.1 \pm 1.2 \times 10^{-4}$
c2 species <sup>a</sup>	c2	JH-20	$5.2 \pm 0.4 \times 10^{-5}$
	bIL67	JH-20	$2.0 \pm 1.2 \times 10^{-4}$
	ml3	JH-20	$3.4 \pm 0.3 \times 10^{-4}$
	eb1	JH-20	$2.2 \pm 0.7 \times 10^{-4}$
P335 species <sup>b</sup>	ul36	JH-23	1.0
	KIT1	JH-23	1.6
	Ø31	JH-23	1.0

Ø50	JH-23	1.0
Q33	JH-23	0.7
Q30	JH-23	0.8
P335	JH-23	0.4

---

<sup>a</sup>EOP of 936 and c2 species is 1.0 on both *L. lactis* subsp. *cremoris* MG1363 (MB112) and MB112 + pLC5 (JH-54). EOP of phages P008 and bIL170 is 1.0 on *L. lactis* subsp. *lactis* IL1403

5 <sup>b</sup>EOP of P335 species is 1.0 on *L. lactis* subsp. *cremoris* (SMQ-86).

To test the  $\phi$ rm for efficiency against P335 phages, the  $\phi$ rm was inserted in a suitable host (SMQ-86) resulting in the strain JH-23. When tested against seven species of P335 phages EOP values around 1 were obtained. To rule out the  
10 possibility that modifications had taken place rendering the  $\phi$ rm inefficient, pJH2 was prepared from JH-23 and re-inserted into MB112 to give strain JH-26. Tests against phage p2 showed an intact  $\phi$ rm phenotype.

Those results showed that the  $\phi$ rm found on the chromosome of *L. lactis* subsp. *cremoris* MG1363 and expressed from pJH2 is effective against phages from most  
15 of the tested 936 species and all tested c2 species while no effect was seen on P335 species.

The results also showed that the  $\phi$ rm encoded by *orf1* is efficient in both the  
20 subspecies (*cremoris* and *lactis*) of *L. lactis*.

Furthermore the results showed that EOP values did not vary whether the  $\phi$ rm was expressed from a promoter located in single copy on the chromosome of the host or from a strong promoter on the vector pJH2. This indicates that the  
25 efficiency of the system is not dependent on the copy number of the gene.

## Example 9

### *Temperature sensitivity*

The efficiency of the  $\phi_{rm}$  was tested against phage sk1 at 30°C and 37°C. EOP values were in both cases around  $10^{-4}$  (data not shown) indicating that the  $\phi_{rm}$  is stable within this temperature range.

## 5 Example 10

### *Type of phage resistance mechanism*

A series of microbiological experiments were conducted to determine the type of  $\phi_{rm}$  encoded by *orf1*.

10

An adsorption assay showed that the level of adsorption of phage p2 to cells with the expressed  $\phi_{rm}$  was  $95.9 \pm 10.6$  % compared to wt MG1363 (data not shown).

- 15 An assay was conducted where the  $\phi_{rm}^+$  strains JH-32, JH-46, JH-47, JH-48 and control MB112 was infected with sk1 that had been fluorescently labeled with the DNA binding dye SYBR-Gold. Following infection the fluorescently labeled phage DNA could be visualized under an epifluorescence microscope. Immediately following phage infection (MOI = 10) of wt strain MB112 a fluorescent halo of
- 20 adsorbed phages was seen surrounding the host cells. Less than 10 min after infection the fluorescent signal on the cell surface was decayed and instead a very bright fluorescent signal was observed in the center of the cell, thus indicating that the phage DNA had been injected into the host cell (data not shown).
- 25 The same result was obtained with the strains having the  $\phi_{rm}^+$  phenotype. This supports the data from the adsorption assay and also shows that the phage DNA is being injected in the  $\phi_{rm}$  containing cells. These results indicate that the  $\phi_{rm}$  is not an adsorption or injection blocking mechanism.
- 30 A cell survival assay showed no increased survival on cells harboring the  $\phi_{rm}$  (Table 3) indicating that the host dies upon infection. The plaque size of phage p2 was smaller when assayed on  $\phi_{rm}^+$  cells compared to  $\phi_{rm}^-$  cells (Table 3). Finally, total DNA extraction from  $\phi_{rm}^+$  cells during a time course experiment of

infection with phage p2 showed phage DNA replication which persisted in the cell throughout the experiment (Fig. 3).

Table 3

Assay	MB112 (wt)	JH-20 (abiV)	5
EOP <sup>a</sup>	1.0	$4.8 \pm 1.8 \times 10^{-4}$	
ECOI (%) <sup>b</sup>	1.0	$0.5 \pm 0.2$	
Burst size (pfu/cell) <sup>c</sup>	$38.8 \pm 5.7$	$11.1 \pm 5.2$	
fraction surviving cells <sup>d</sup>	$6.1 \pm 1.3 \times 10^{-5}$	$3.1 \pm 0.3 \times 10^{-6}$	10
phage DNA replication <sup>e</sup>	+	+ (concatemeric)	
plaque size (mm)	1.5 - 1.7	pinpoint - 0.7	

<sup>a</sup>n=3

15 <sup>b</sup>MOI = 0.5, n=3

<sup>c</sup>MOI = 0.2, n=3

<sup>d</sup>MOI = 5, n=3

<sup>e</sup>MOI = 2, n=1

20 All the above results confirm that the mechanism is a  $\phi$ rm that functions as an abortive infection mechanism. This was named AbiV.

### Example 11

*Sequence analysis of the 1.3 kb DNA fragment containing the  $\phi$ rm*

25

The DNA fragment cloned in pJH2 was sequenced (SEQ ID NO 3 and 5). The fragment consists of 1300 nucleotides. Nucleotides 1 to 1300 correspond to nucleotides 1021 to 2320 in the Lin sequence (GenBank acc.nr: AF324839). One significant open reading frame (orf) was found encoding the polypeptide

30 sequence shown in SEQ ID NO 1. This gene encoding the  $\phi$ rm was named *abiV* (SEQ ID NO 3) and the translated protein was named AbiV (SEQ ID NO 1). The G+C content of the gene was found to be 31.7%. This value is typical for abi mechanisms which are known to have lower G+C contents compared to the normal 37% in *L. lactis*. Searches for promoter sequences upstream of *abiV* (bp 1

to 430) were performed but no suitable promoter could be found in this region. This corresponds well with the hypothesis of the  $\phi$ rm being silent in the wt strain MB112. The translation start codon was preceded (8 bp upstream) by a ribosome binding site (5'-TGAACGGAGAG-3', underlined sequence matches consensus sequence).

## Example 12

### *Analysis of the AbiV protein encoded by abiV in pJH2*

Since the *abiV* gene is the only orf in the cloned sequence of pJH2 and a frame shift mutation in this orf causes the phage sensitive phenotype, it is concluded that the protein encoded by this gene is responsible for the  $\phi$ rm<sup>+</sup> phenotype. AbiV consists of 201 amino acids and has a molecular weight of 22692 Da. The pI was estimated to be 5.37.

15

The protein does not contain any putative transmembrane or signalpeptide motifs and it is therefore likely that the protein is cytosolic. Homology searches in databases did not reveal any homology (at amino acid or nucleotide level) to other lactococcal proteins or any proteins with known function. Likewise, no

20 conserved domains were found in the protein.

The deduced function of AbiV is therefore new and the  $\phi$ rm is a novel abi mechanism.

## 25 Example 13

### *Effect of AbiV on phage life cycle*

The effects of the AbiV system was tested on the phage p2 life cycle using the phage sensitive strain MB112 and the corresponding AbiV containing strain JH-20. The following results are summarized in table 3.

30

The propagation of p2 on JH-20 was inhibited as seen by the EOP of ca  $10^{-4}$  and the plaque size was reduced from about 1.5 mm to <1 mm. Very few of the infected cells harboring the  $\phi$ rm survived infection.

- 5 The ECOI on JH-20 was  $0.5 \pm 0.2$  % indicating that only 5 out of 1000 infected cells managed to release at least one viable phage. In these successful infections the burst size was reduced by 72 % (from  $38.8 \pm 5.7$  in MB112 to  $11.1 \pm 5.2$  in JH-20).
- 10 The combined negative effects of AbiV on cell survival, ECOI and burst size were the cause of the reduced plaque size and EOP of p2 on JH-20.

- 15 The replication of phage DNA was followed in a 2h phage infection experiment of p2 on phage resistant JH-20 and phage sensitive MB112 (Fig. 3). Phage DNA was visualized by digesting the total DNA prepared from an infected cell culture with *EcoRV* and comparing the resulting fragments run on an agarose gel with the *EcoRV* restriction map of phage p2.

- Ten minutes after infection replication of phage DNA was observed in both strains.
- 20 In MB112 the concentration of phage DNA decreases around 40 min after infection coinciding with lysis of the sensitive host culture. On the contrary, in JH-20 the phage DNA persists throughout the experiment which was terminated after 2h. Inspecting the *EcoRV* digested phage DNA fragments, two bands of 1.3 and 4 kb respectively and a 5.3 kb fragment are seen in the phage sensitive culture. The
- 25 5.3 kb fragment is spanning the *cos* site on the phage DNA which is the site where the replicated phage DNA is cut into identical units of complete phage genomes before packaging of the DNA into the capsids. Therefore the 1.3 and 4 kb fragments represent DNA that has been cut at the *cos* site. The presence of both non-resolved and resolved DNA in the phage sensitive strain is due to the
- 30 continuous DNA replication throughout the phage life cycle and the simultaneous packaging of already resolved DNA into the phage capsids. In JH-20 (AbiV<sup>+</sup>) only the 5.3 kb fragment is observed which indicates that the phage DNA is not cut at the *cos* site in this strain.

The above results show that AbiV works after phage DNA replication and is thus categorized as a late abi mechanism. The presence of concatemeric DNA fragments (*cos* site not cut) further suggests that the  $\phi$ rm might work at a late stage for example during packaging of phage DNA into the capsids.

5

### Example 14

#### *Phage genes involved in sensitivity to AbiV*

A number of phage mutants capable of overcoming AbiV were isolated. On JH-20  
10 AbiV-insensitive mutants of p2, sk1, jj50 and c2 were isolated and named p2.1, sk1.1, jj50.1 and c2.1, respectively. On JH-22, mutants of P008 and bIL170 were isolated and named P008.1 and bIL170.1, respectively.

The full genome of mutant p2.1 was sequenced revealing only mutations in the  
15 region around the early gene *orf26* (SEQ ID NO 4). SEQ ID NO 4 encodes a polypeptide sequence denoted SEQ ID NO 2. The following polynucleotide mutations were found in phage p2.1 that escaped the AbiV-mechanism:

- Two point mutations in *orf26* leading to amino acid changes.
- One point mutation in the intergenic region between *orf26* and the  
20 upstream gene *orf27*
- A 55 bp deletion including the startcodon and 6 downstream base pairs of *orf26*.

The homologues of p2 *orf26* in the other phage mutants were sequenced.  
25 Nonsense mutations were observed in: *orf26* (sk1.1), *orf25* (jj50.1), *orf33* (P008.1), *e24* (bIL170.1) and a point mutation leading to an amino acid change (T to P) was seen in *e11* (c2.1).

These data show that AbiV-resistant phage mutants apparently fail to produce  
30 functional protein encoded by an early gene homologous to phage p2 *orf26*. In at least one phage mutant (p2.1), *orf26* is the only gene which is mutated. Finally, phage 712 (936 species) is the only phage among the tested phages from the 936 and c2 species that does not contain an *orf26* homologue. Among the wt phages

of the 936 and c2 species, this phage is also the only one which is not sensitive to AbiV.

Based on the above results, it is concluded that a functional copy of phage p2  
 5 *orf26* (and homologues in other phage species) is mandatory for successful  $\phi$ rm<sup>+</sup>  
 phenotype of AbiV. The gene is named *sav* (sensitivity to abiV) and the translated  
 putative protein was named Sav. It is thus possible to strengthen the AbiV-  
 mechanism by supplying the AbiV host cell with a polynucleotide sequence  
 encoding wt Sav.

10

A nucleotide blastn analysis of phage p2 *orf26* revealed a high degree of  
 sequence homology to other lactococcal phage genes: jj50 *orf25* (99.7%), sk1  
*orf26* (99.0%), P008 *orf33* (91.4%), bIL170 *e24* (90.6%). Furthermore the  
 translated p2 *orf26* showed a more distant relationship (29%) with phage c2 gene  
 15 *e11*. Despite the low degree of homology the *e11* gene of phage c2 is involved in  
 sensitivity to AbiV since a mutation in this gene helps the phage c2.1 escape AbiV.  
 Therefore, sequences of either phage 936-like *orf26* homologues (SEQ ID NO 2)  
 or c2-like *e11* homologues (SEQ ID NO 7; DNA sequence: SEQ ID NO 8, derived  
 from accession number NC001706 disclosing the complete genome of *Lactococcus*  
 20 *lactis* phage c2), or variants or fragments thereof are a part of the present  
 invention.

### Example 15

*Analysis of the phage p2 gene orf26 (sav) and the putative protein (Sav) encoded*  
 25 *by this gene*

The DNA fragment containing phage p2 gene *orf26* and the upstream intergenic  
 region to *orf27* was sequenced on both strands. The sequenced fragment contains  
 499 nucleotides (SEQ ID NO 5). The *sav* gene consists of 384 bp (SEQ ID NO 4).  
 30 Upstream of *sav* in a suitable (8 bp) distance is found a RBS sequence  
 (GGATTGGGGGT, underlined sequence matches consensus sequence). No  
 promoter sequence is found in the region between *orf27* and *sav*. This  
 corresponds well with the genetic structure of this region in p2 and in the closely



related phage sk1. In both phages *orf26* is the last gene in a putative operon consisting of *orf30* to *orf26* where the promoter is upstream of *orf30* (4).

The *sav* gene is located at the end of the early transcribed region of phage p2.

- 5 The putative protein Sav (SEQ ID NO 2) encoded by the gene *sav* consists of 128 amino acids. It has a theoretical molecular weight of 15.3 kDa and an estimated pI of 4.62. Homology searches revealed homology to a number of putative proteins in related phages of the 936 and c2 species. However, no homology was found to proteins with known function. Nor was found any conserved domains in
- 10 the protein. The protein is thus new and it has not previously been associated with sensitivity to phage resistance mechanisms. SEQ ID NO 7 is present in the database under accession number NC001706 and it has not previously been associated with sensitivity to phage resistance mechanisms.
- 15 The interaction of Sav with AbiV is not known but the insensitivity to AbiV of phages with a deleted *sav* gene clearly indicates that *sav* is involved in sensitivity of the phage to the  $\phi$ rm.

- 20 Co-expression of *abiV* and *sav* in host cells will most likely enhance the efficiency of AbiV since the escaping mutant phages will have to mutate in other genes than *sav*. Co-expression might also broaden the range of phages against which AbiV is effective. These are so far only hypotheses but they are in the process of being tested experimentally.

- 25 Since *sav* has not previously been associated with any  $\phi$ rm, the AbiV  $\phi$ rm in the present invention is a new abi mechanism interacting in a so far unknown way with the sensitive phage. AbiV is therefore likely to be an efficient  $\phi$ rm capable of supplementing already isolated and used phage resistance mechanisms thus improving the field of phage resistance mechanisms.

30

- The discovery of a phage gene involved in sensitivity to the Abi-resistance mechanism may be used for obtaining a phage resistance mechanism that is more efficient than use of the AbiV-mechanism alone. It is thus likely that the use of the wild type *orf26*-sequence encoding the polypeptide according to SEQ ID NO 2
- 35 and/or SEQ ID NO 7 will fully or partly prevent that the phage can escape the Abi-

mechanism according to the present invention by supplying AbiV-sensitive protein (SaV) together with AbiV-protein.

The present invention thus relates to the use of polynucleotide sequences  
5 encoding both SEQ ID NO 1 and SEQ ID NO 2 and/or SEQ ID NO 7 (or a variant thereof) within a *Lactococcus* cell in order to exploit the synergy that exists in this combination. Compared to other known Abi-systems, the combination of SEQ ID NO 1 and SEQ ID NO 2 and/or SEQ ID NO 7 (or a variant thereof) in the same cell provides for a phage resistance mechanism that is extraordinarily efficient in  
10 preventing phage infections and thus preventing the emergence of AbiV-resistant phages.

### **Example 16**

*Use of bacteria according to the invention*

15

The  $\phi$ rm according to the present invention can be used in connection with dairy starter cultures in existing dairy production plants to produce any fermented dairy food product.

### **20 Example 17**

*Construction of expression vector pLC5*

The pGKV259 vector (18) was used as the starting molecule from which pLC5 was derived. pGKV259 was digested with *Pst*I (located downstream from the P59  
25 promoter) followed by gel purification. Two complementary oligonucleotides (5'-TGGATCCAAAGGAGGTCCTGCA-3' and 5'-GGACCTCCTTTGGATCCATGCA-3') were annealed together using standard procedures (16) to create a double stranded linker with *Pst*I-compatible sticky ends. This linker also contained a unique *Bam*HI site and a ribosome binding site (RBS: 5'-AGGAGG-3'). The linker was inserted  
30 into the *Pst*I site of pGKV259 and the ligation mixture was transformed into *E. coli* MC1061. Transformants were selected on LB plates containing 20µg/ml chloramphenicol. Positive clones with the linker inserted in the right direction were identified by colony PCR. Correct clones were later confirmed by sequencing.

Upon introduction of the linker into pGKV259, the PstI site on the 5'-side of the linker was disrupted whereas the one on the 3'-side was conserved. Thus, a unique PstI site was created 8-bp downstream from the RBS. Cloning of an insert harboring its own ATG start codon into the PstI site of pLC5 enables efficient transcription from the P59 promoter, and translation from the introduced RBS. For this invention, however, the native RBS of AbiV and not the RBS in the vector was used for translation of the protein.

### Example 18

10

#### *RNA isolation, purification and RT-PCR analysis of transcription*

Overnight cultures were diluted 100-fold and grown to  $OD_{600} = 0.5$  at 37°C. Aliquots (2 ml) were harvested by quick centrifugation (20,000 g, 30 sec) and the pellet was resuspended in a solution of 0.5 M sucrose with 60 mg/ml lysozyme. Following incubation (37°C, 15 min), the cells were pelleted and resuspended in 1 ml TRIzol Reagent (Invitrogen). Total RNA was isolated according to the manufacturer's instructions. Prior to reverse transcription (RT)-PCR, the RNA samples were treated with the DNase based TURBO DNA-free kit (Applied Biosystems).

RT-PCR was carried out using the RevertAid First Strand cDNA Synthesis kit (Fermentas) as recommended by the manufacturer. As a control, the RT-PCR procedure was carried out without reverse transcriptase to ensure that the RNA samples were free of contaminating DNA.

### Example 19

#### *30 Mutants of L. lactis subsp. cremoris MB112 spontaneously expressing AbiV*

Cultures of *L. lactis* MB112 in exponential growth were mixed with the lytic phage sk1 (MOI > 1) in presence of 10 mM  $CaCl_2$  and incubated 10 min at room temperature before plating and incubation at 36 °C overnight. Spontaneous mutants were observed with a frequency of ca.  $10^{-8}$ . Forty single colonies were

purified and cross-streaked against phages sk1, p2, 712 and p2.1. A bacterial mutant expressing AbiV is expected to be resistant to sk1 and p2 but sensitive to 712 and p2.1 (Table 1 and Table 2). Possible candidates were tested with EOP for resistance to phages p2, 712, p2.1. One mutant (JH-80) revealed the expected  
5 pattern of a mutant expressing AbiV with EOP values of  $2 \times 10^{-5}$ , 0.75 and 0.8, respectively.

This mutant was investigated for transcription of the *abiV* gene using reverse transcriptase PCR (RT-PCR) (Fig 6), as described in example 18.

10

These results demonstrate that it is possible to obtain mutants of *L. lactis* strains carrying *abiV* on the chromosome which spontaneously express AbiV. This experiment demonstrates that it is possible to obtain phage resistant bacteria expressing AbiV without using genetic modification. This is particularly interesting  
15 for the dairy industry that prefers to avoid the use of genetically modified organisms (GMO).

## Example 20

### 20 Conjugal transfer of *abiV*

In order to improve the non-GMO alternative of the  $\phi$ rm invention a conjugation experiment was conducted in which the  $\phi$ rm was transferred from the chromosome of JH-32 (donor) to MG1614 (recipient) (Table 4). Briefly, donor and  
25 recipient were recovered from plates and mixed at high cell densities ( $OD_{600} = 40$ ). After 2 min incubation the cell mixture was plated on non-selective plates and incubated overnight in anaerobic conditions. The cells were then recovered from the plates and plated with selection for donor (erythromycin resistance), recipient (rifampicin resistance) and transconjugants (erythromycin and rifampicin  
30 resistance), respectively.

Since the erythromycin resistance gene is inserted just upstream of *abiV* in JH-32 the erythromycin resistance phenotype was used to select for transfer of this gene to MG1614 hoping that *abiV* would be transferred along with it. Rifampicin  
35 resistance was used to select for MG1614.

A number of transconjugant candidates were isolated and purified. The additional phenotypes (resistance to phage, streptomycin and fluorouracil) were used to test the isolated candidates for verification of the phage+erythromycin resistance from JH-32 to MG1614. In JH-81 the expected pattern was observed (Table 4). An EOP value of  $10^{-4}$  which is similar to other EOP values obtained with AbiV (Table 2) makes it plausible that *abiV* was transferred and expressed in MG1614.

This experiment demonstrated that it is possible to transfer AbiV by conjugation from one bacterium to another. Conjugation is not considered as genetic modification and the method is thus suitable for the industry for transferring AbiV between bacterial strains in a non-GMO manner.

Table 4

15

Phenotype of donor (D), recipient (R) and transconjugant (T). Selection for transconjugants was done using erythromycin and rifampicin.

Resistance	Phage	Erythromycin	Rifampicin	Streptomycin	Fluorouracil
JH-32 (D)	yes	yes	no	no	yes
MG1614 (R)	no	no	yes	yes	no
JH-81 (T)	yes	yes	yes	yes	no

20

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**Claims**

1. An isolated polynucleotide sequence that encodes a polypeptide with at least 70% identity with SEQ ID NO 1, or a fragment thereof, and wherein expression of  
5 said polynucleotide confers at least one phage resistance mechanism to a *Lactococcus* bacterium.
2. An isolated polynucleotide that encodes a polypeptide with at least 70% identity with a sequence selected from the group consisting of SEQ ID NO 2, or a  
10 fragment thereof, and SEQ ID NO 7 or a fragment thereof and wherein said polynucleotide optionally furthermore encodes a polynucleotide according to claim 1, and wherein expression of said polynucleotide confers at least one phage resistance mechanism to a *Lactococcus* bacterium.
- 15 3. An expression vector encoding at least one of the polypeptides according to claims 1 and 2.
4. A *Lactococcus* bacterium comprising an expression vector according to claim 3.
- 20 5. A starter culture composition comprising the bacterium according to claim 4.
6. An isolated polypeptide conferring at least one phage resistance mechanism to a *Lactococcus* bacterium, wherein said polypeptide is selected from one or more of the group consisting of: a polypeptide with at least 70% identity with SEQ ID NO  
25 1, or a fragment thereof, a polypeptide with at least 70% identity with SEQ ID NO 2, or a fragment thereof, and a polypeptide with at least 70% identity with SEQ ID NO 7 or a fragment thereof.
7. Use of one or more polynucleotides according to any one of claims 1 or 2 or  
30 one or more polypeptides according to claim 6 for improving phage resistance in a *Lactococcus* bacterium.
8. A method for fermenting a food product, said method comprising the step of adding one or more of the components selected from the list consisting of: an

expression vector according to claim 3, a bacterium according to claim 4, a starter culture according to claim 5, and a polypeptide according to claim 6 prior to fermentation of said food product.

5 9. A food product obtainable by a method according to claim 8.

10. A method for obtaining phage resistant bacterial cells, said method comprising use of pGhost9::ISS1 for random insertion into a bacterial cell and subsequently screening and selecting for phage resistant cells.

10

11. A phage resistant cell with improved phage resistance obtained by the method according to claim 10.

12. A cell according to claim 11, wherein a polynucleotide sequence encoding SEQ  
15 ID NO 1 is transcriptionally active and wherein the cell is a *Lactococcus* bacterium.

13. A *Lactococcus* bacterium that expresses at least one polypeptide selected from the group consisting of: a polypeptide with at least 70% identity with SEQ ID NO 1, or a fragment thereof, a polypeptide with at least 70% identity with SEQ ID NO  
20 2, or a fragment thereof, and a polypeptide with at least 70% identity with SEQ ID NO7, or a fragment thereof.

**Abstract**

The present invention relates to the field of dairy science. In particular, the present invention relates to methods for improving dairy starter culture quality as well as food products that can be obtained using such methods.

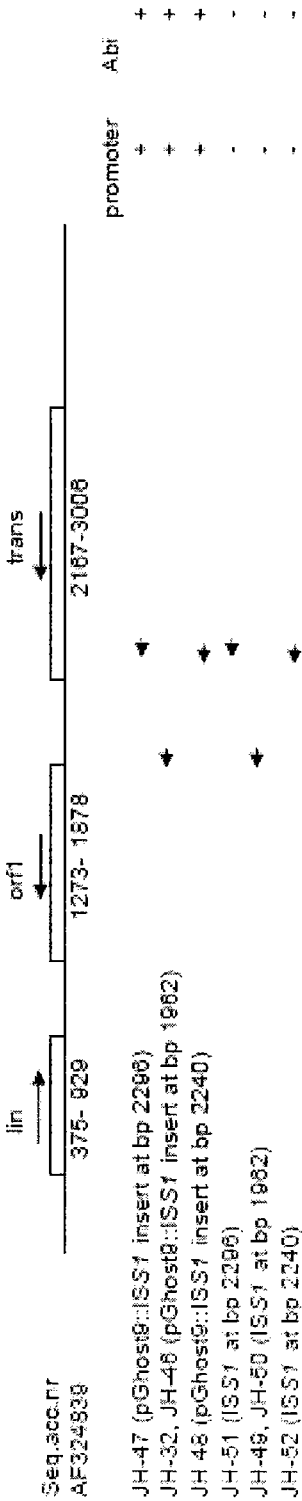
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1/6

Name	Source	Seq. Acc. Nr	Author	seq. publ	Patent nr	Sensitive phages	EOP (range)
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AbiB	pHP003	AF247159	Cluzel et al	2000	WO9205260-A	936	?
AbiC	pTN20	M95956	Durmaz et al	1994		936, P335	1E-2 to 1E-4
AbiD	pBF61	U10992	McLandsborough et al	1995		936, c2	1E-4
AbiD1	pIL105	L35176	Bidnenko et al	1995		936, c2	1E-4 to 1E-6
AbiE	pNP40	U36837	Garvey et al	1995		936	1E-4
AbiF	pNP40	U36837	Garvey et al	1995		936, c2	1E-4 to 1E-6
AbiG	pCI750	U60336	O'Connor et al	1996	US5019506-A	936, (c2), P335	1E-3
AbiH	chromosome?	X97651	Prevots et al	1996	US5955332-A	936, (c2)	?
AbiI	pND852	U38973	Su et al	1997	DE19538001-A1	936, (c2)	?
AbiJ	pND859	U41294	Deng et al	1995	DE19538001-A1	(936)	?
AbiK	pSRQ800	U35629	Emond et al	1997	EP868514-A	936, (c2), P335	1E-5 to 1E-8
AbiL	pND861	U94520	Deng et al	1999	DE19538001-A1 ?	936, (c2)	?
AbiN	prophage	Y11901	Prevots et al	1998	US6712150-A	936, c2	?
AbiO	pPF144	I61427	Prevots et al	1997	US 5658770	936, c2	?
AbiP	pIL2614	U90222	Domingues et al	1998		936	1E-7
AbiQ	pSRQ900	AF001314	Emond et al	1998	WO9928476-A	936, c2	1E-8
AbiR	pKR223	AF216814	Twomey et al	2000		c2	?
AbiT	pED1	AF483000	Bouchard et al	2002	WO200281697-A	936, P335	1E-5 to 1E-7
AbiU	pND001	AF188839	Dai et al	2001		(936), c2, (P335)	1E-1 to 1E-2
AbiZ	pTR2030	U17233	Durmaz and Klaenhammer	2006	US2005130126-A1	P335	1E-6
AbiV	chromosome and pLC5		Haaber et al	2007		936, c2	1E-4

Fig. 1

A



B

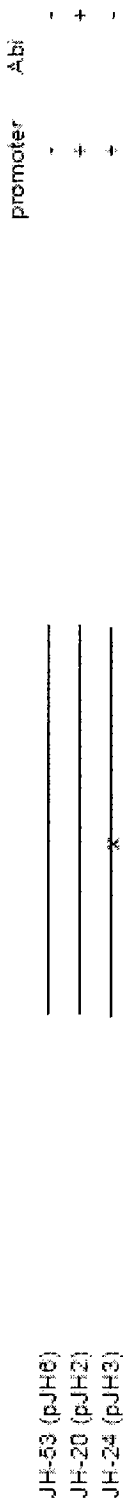


Fig. 2

3/6

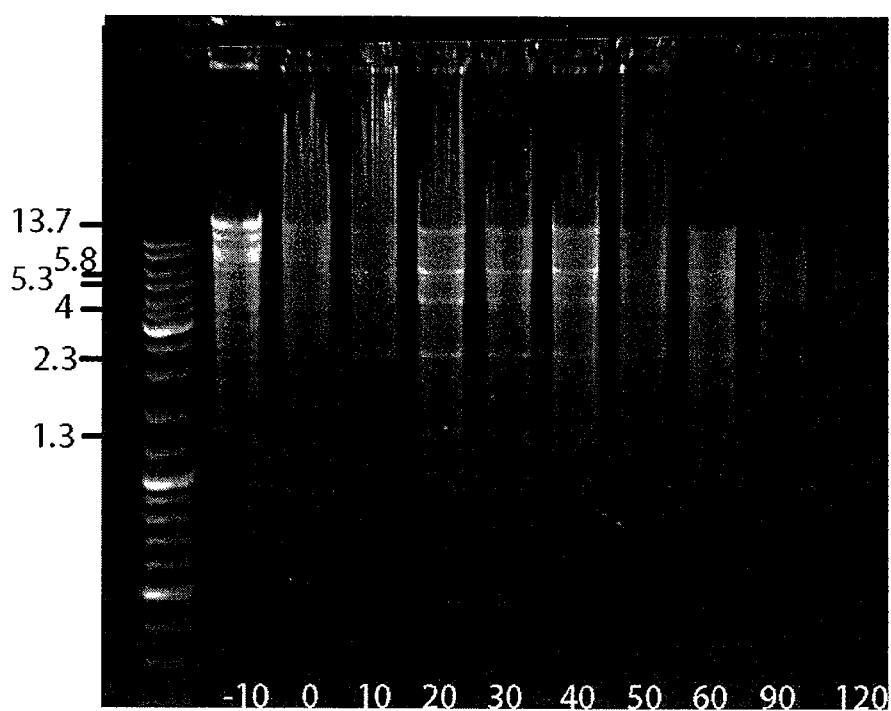
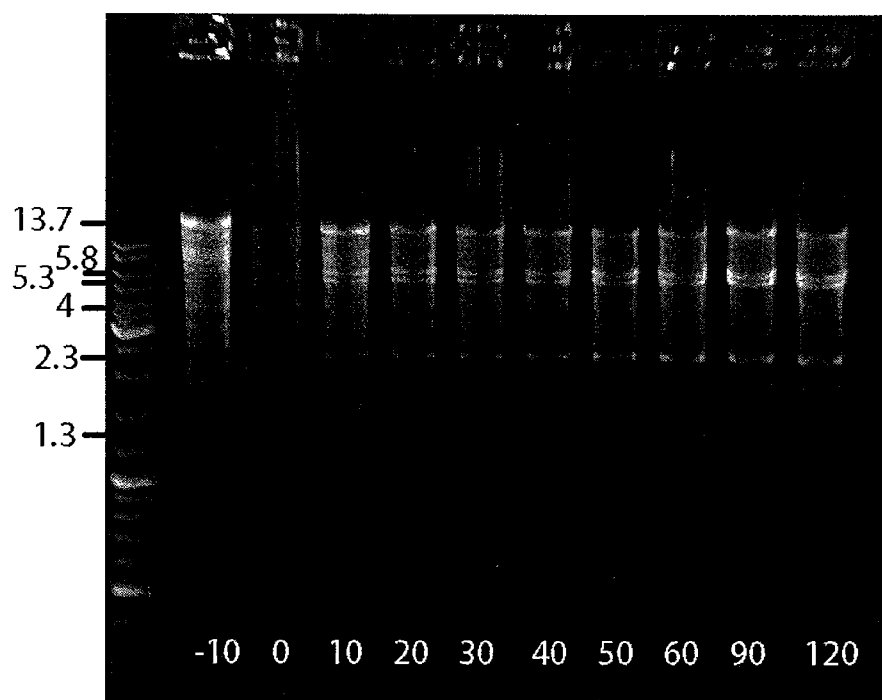


Fig. 3

4/6

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-
121 - TACACCTAATCAATTTGAAAAGGTAAGTGCTTAAATAAATAGATTAAAAATTCTACGTTT - 180
-
181 - GTTACTCTAAAAACTTGACTTAAACGTCACCTTCTTGAGTTAACTTCGCATAATAAAGAAAA - 240
-
241 - CAAAGACTTTTTGGATGAAATAGACAAAACCTTACTCTAAAAATTGATACTGTCAATACTAA - 300
-
301 - AGTTAGACAAAACGAAGTAGCTGCAACTACTAATCAACTTGGCGCTAACTAAAGCAAATGT - 360
-
361 - ACAAAATTCATACCCTTTTAGTAATTGCTAGTAATTATTATCAATCAGTATGGATCCAGAT - 420
-
421 - TAAAGAATGAACGGAGAGTTTTATGTTTGATAAAGACAACCTATGCATTAGGAAAAATGAA - 480
1 -      RBS      M F D K D N Y A L G K M K - 13
481 - GAATACCCTTAATACCAAAGAAAAGTAAGTTTTCTCTAAAGTCAACTGATGATCTTAATAA - 540
14 - N T L N T K E S K F S L K S T D D L N K - 33
541 - ATGCATCGATCATATTTTCAGTCTTAATAAAAGATGCATATCTGCTTTATACGAATGAATC - 600
34 - C I D H I S V L I K D A Y L L Y T N E S - 53
601 - ATTTGCCCACTTCTACATTCATTTCAATAACAATTATTGAAGAAGTTGGTAAAACTCATAT - 660
54 - F A T S T F I S I T I I E E V G K T H I - 73
661 - AGGTATGTTTATCAGTGAGAATAAAGATATAAAGCGTGGGAAAGACCCTTTGAGAAATCA - 720
74 - G M F I S E N K D I K R G K D P L R N H - 93
721 - TAAATCCAAACACGCTTTTGGATCTCTTCCAACCTATAAAAAATGGGAGGACGACTTAATAA - 780
94 - K S K H A F G S L P T I K M G G R L N K - 113
781 - GGCTATTGGAGATGAAATGATTGATAAAATCGTCGAAGATGCCGAAACTGGTGAACCTAT - 840
114 - A I G D E M I D K I V E D A E T G E L I - 133
841 - TTCAATACGGGAGTCATCTTTGTATGCAGATATTATTGATGATATTCTTGAAGTACCTAG - 900
134 - S I R E S S L Y A D I I D D I L E V P S - 153
901 - TGAAAAAATTAGTAAAGAACAAAGTAGAGCATTGCTCCTTTATGCGATAGAATGTTTTGA - 960
154 - E K I S K E Q S R A L L L Y A I E C F D - 173
961 - TGACAGTTTAGTTGGCTATACACATCATTTCATTTGAAGTATCAGAGACAACTGATGAGTT - 1020
174 - D S L V G Y T H H S F E V S E T T D E L - 193
1021 - GTTTGAAAAGTTAGCAAACAATAAATAGTTAAATCTTGAGTTTGATTTTGCTGAATATTC - 1080
194 - F E K L A N N K *
1081 - TGCATTTATCGGGCGGAATGATGCCCTTAGACTTTGCAACAGAACCTCGATTTTAATTGG - 1140
1141 - TTCAGAATAGGTTATACTAGACAAAAGATCGGCTCCTAAAAATGGGTTTGTGATAAACAC - 1200
1201 - CATTTTAAAGGAAGCTGGTCTTTTTTGTCCAAACACTGGTCAGACAATTTTGGGGCCTAT - 1260
1261 - GATATTTGGTGTGATAGATAAAAATTCATCAACACTATTC - 1300

```

Underlined sequence is Ribosome Binding Site (RBS)

Fig. 4



5/6

**orf26 (sav) and upstream region after orf27 stop codon**

```

1 - AAGATACAGTAAAAAAGCTTTAATGATAGCTGTAGGTATAGGCTTTACACTTATCGCTATCA - 60
-
61 - CTTGGATAGGTATAATTGCAACGTTGCTTATTACATGGATTGGGGGTAAACATCTAATGAA - 120
1 -                                     RBS                               M N - 2

121 - TTATGGTACAAAATAAGCACTATGCCAATGAATACGGTATGGAACTTAACGAATACTTTAA - 180
3 - Y G T N K H Y A N E Y G M E L N E Y F K - 22

181 - ACATCATTTTATAGCTATGAAAGAGCTTGCAAGGCTGGTATACAATGCAGGTATTAAAGTATCT - 240
23 - H H F S Y E E L A G W Y T M Q V L K Y L - 42

241 - AGTGAGAGCTGGCAAGAAAAGAGGGTGAAAAGCTACGACAAAAGACCGTAACAAGGCTTTAGA - 300
43 - V R A G K K E G E S Y D K D R N K A L D - 62

301 - CTATGCAGGAGAACTTGCTAACTTAAGTAACGAGAATGAGCTTACAGAATACACTACTGA - 360
63 - Y A G E L A N L S N E N E L T E Y T T D - 82

361 - CGACATTATGGGCTTTGCACAAAGATATAGCTGATGATTTCAAACAATGGAAAAGGCGAAAAG - 420
83 - D I M G F A Q D I A D D F K Q W K G E R - 102

421 - AAATAAAGTTTAAATCAGAGTTTCACGAAAAGAAGAGATAAAAAGCGATTGATGAAAAGATACTT - 480
103 - N N F K S E F T K E E I K A I D E R Y L - 122

481 - GGAATTTATTGAAAGAGGTC - 499
123 - E F I E E V - 128

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Fig. 5

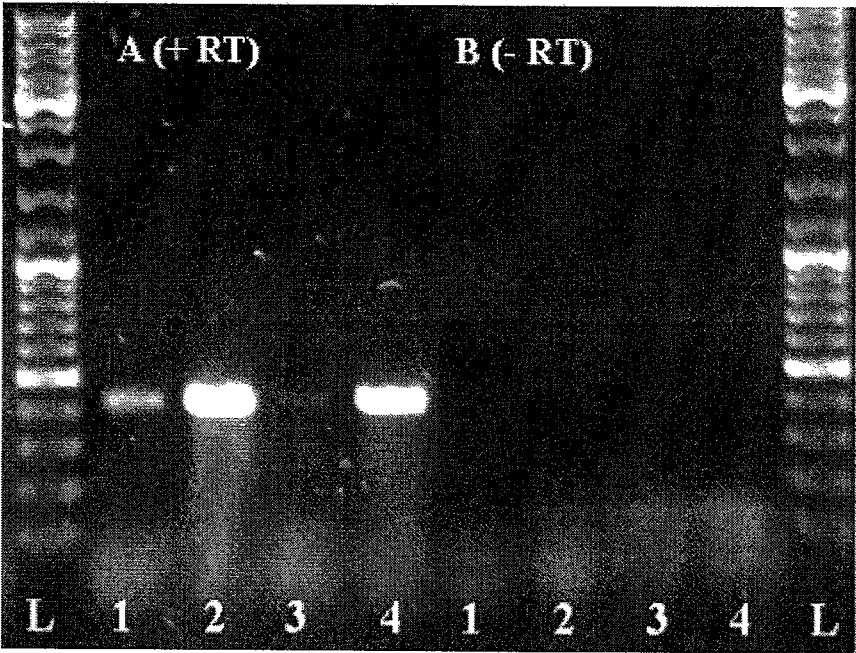


Fig. 6

Sequence listing ver 3 P42448EP01.ST25  
SEQUENCE LISTING

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Haaber, Jakob Brandt Borup

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35 40 45

Tyr Thr Asn Glu Ser Phe Ala Thr Ser Thr Phe Ile Ser Ile Thr Ile  
50 55 60

Ile Glu Glu Val Gly Lys Thr His Ile Gly Met Phe Ile Ser Glu Asn  
65 70 75 80

Lys Asp Ile Lys Arg Gly Lys Asp Pro Leu Arg Asn His Lys Ser Lys  
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His Ala Phe Gly Ser Leu Pro Thr Ile Lys Met Gly Gly Arg Leu Asn  
100 105 110

Lys Ala Ile Gly Asp Glu Met Ile Asp Lys Ile Val Glu Asp Ala Glu  
115 120 125

Thr Gly Glu Leu Ile Ser Ile Arg Glu Ser Ser Leu Tyr Ala Asp Ile  
130 135 140

Ile Asp Asp Ile Leu Glu Val Pro Ser Glu Lys Ile Ser Lys Glu Gln  
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Ser Arg Ala Leu Leu Leu Tyr Ala Ile Glu Cys Phe Asp Asp Ser Leu  
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Sequence listing ver 3 P42448EP01.ST25

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Glu Gly Glu Ser Tyr Asp Lys Asp Arg Asn Lys Ala Leu Asp Tyr Ala  
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Gly Glu Leu Ala Asn Leu Ser Asn Glu Asn Glu Leu Thr Glu Tyr Thr  
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Sequence listing ver 3 P42448EP01.ST25

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Phe Thr Thr Gly Gln Gly Phe Ala Leu Thr Asn Ala Leu Lys Tyr Thr
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ggaatttatt gaagaggtc	499

# **DISCUSSION & OUTLOOK**



## Discussion and outlook

The results of my PhD project have revealed a novel Abi mechanism and provided data to describe the mode of action (MOA) and industrial applicability of this system. The results have been discussed in the accompanying manuscripts and this discussion is thus meant as a summary and a supplementary discussion. Finally, perspectives of the findings and suggestions for further work are presented.

### DESCRIPTION OF THE ABIV PHAGE RESISTANCE SYSTEM

The novel lactococcal abortive infection mechanism AbiV consists of the bacterial protein AbiV and the phage protein SaV. Both proteins were isolated and characterized during my 3-year PhD study. The proteins interact after phage infection at an early stage of the lytic cycle to inhibit phage protein synthesis in the host cell whereby further phage proliferation is halted. Below, a description of the individual components of the system as well as more detailed discussion of their interaction is presented along with suggestions for the industrial applications of the system.

#### Characteristics of the bacterial protein AbiV

AbiV is a newly discovered and characterized lactococcal protein that confers resistance against 936-like and c2-like phages when expressed in *L. lactis* (Manuscript I). The phenotypic effects on cells expressing AbiV during a phage infection are normal adsorption and transfer of phage DNA into the host cell along with death of the cell with very limited release of infective phages. These are all well known characteristics of an Abi mechanism and AbiV was concluded to be a novel Abi protein. A frameshift mutation in the *abiV* gene abolished the phage resistance phenotype, indicating that a translated AbiV is responsible for the phage resistance phenotype. The protein consists of 201 amino acids and it was shown in several independent experiments to form a dimer in the native form that most probably exists in the cytosol of the cell (Manuscript I and III). With only few common characteristics of Abi proteins<sup>26</sup>, such basic structural information might be of value. However, to date AbiV is the only Abi protein for which the multimeric state has been revealed. AbiV contains a putative RNA binding site<sup>145</sup> which might be involved in the systems mode of action as discussed below. AbiV, like most Abi proteins showed no amino acid similarity to known proteins or contained any conserved domains when database searches were performed, which

prevented us from doing predictions on the MOA of the system<sup>26</sup>. The majority of the isolated Abi systems are encoded by a single plasmid borne gene though a few Abi systems are encoded by two<sup>11,32,35,59,117</sup> or more<sup>150</sup> genes. AbiV is encoded by a single gene with the characteristic low G+C content and it is located in the “integration hot spot” region of *L. lactis* MG1363 chromosome<sup>154</sup>. This region is characterized by a high concentration of remnants of mobile DNA, which makes it tempting to speculate that a plasmid borne *abiV* previously integrated in this region. Contrary to the other chromosomally encoded Abi systems, *abiV* is not expressed from the chromosome of wild type *L. lactis* MG1363 (Manuscript I). Though both *abiH*, *abiN*, and *abiB* are expressed from the host chromosome<sup>124,126,28</sup>, the over-representation of plasmid encoded Abi systems, indicates that expression of chromosomal Abi genes is not a common strategy in *L. lactis*. However, Chopin *et al.* argued that the under-representation of isolated chromosome-encoded Abi systems could be due to the methodological advantages in isolating phage resistance mechanisms from plasmids<sup>26</sup>.

AbiV was discovered and isolated using the insertional mutagenesis system pGhost9::ISS1<sup>99,100</sup>, which is normally used in experiments where selection is made for loss-of-function effects<sup>47,95,127,147</sup>. However, Luccini *et al.* isolated phage resistant mutants of *S. thermophilus* where the insertion conferred upregulation of a downstream gene encoding a R/M phage resistance mechanism<sup>95</sup>. A similar property of the pGhost9::ISS1 system caused upregulation of *abiV* and led to the discovery and isolation of the *abiV* gene.

Though the ISS1 insertion sequence is reported to integrate randomly on the chromosome of *L. lactis*<sup>100</sup>, we observed that in several independently obtained mutants, ISS1 had integrated within a 350 bp region of the 2.5 Mbp chromosome of *L. lactis* MG1363. In Manuscript I, we suggest that insertion sequences of the ISS1 type may integrate into the 59-kb “integration hot spot” on the *L. lactis* MG1363 chromosome with a high frequency compared to other parts of the chromosome based on the high concentration of insertion sequences in this region (almost 20% of the 71 insertion sequences found in the MG1363 genome). Thus ISS1 integration in MG1363 appears to be not completely random with a preference for insertion in the integration hot spot region which could explain the observed high frequent integration events upstream of *abiV*.

### **Possible function of the early phage gene *sav***

To analyze the genetic component(s) of the phage that interact with AbiV to cause the phage resistance phenotype, we took advantage of the ability of different lactococcal phages to form AbiV insensitive mutants (Manuscript II). The complete genome of one of these phage mutants (p2.1) was

sequenced and compared to the wild revealing several mutations in a small part of the early transcribed region of the phage. Sequencing of this region in other AbiV-insensitive phage mutants revealed that a gene which is transcribed early in the wild type phage was involved in sensitivity to AbiV. The gene was named *sav* for sensitivity to AbiV. In total, the *sav* gene homologue was investigated in four phage mutants of the 936 species (p2.1, sk1.1, bIL170.1, and P008.1) and two mutants of the c2 species (c2.1 and bIL67.1).

Most of the observed mutations in the *sav* homologues of the six investigated phage mutants effectively prevented translation of the SaV protein (nonsense mutations, deletion of start-codons) apparently without causing significant fitness loss for the phage (Manuscript II). SaV thus seemed to be redundant in the p2 lytic cycle. Phages are normally considered to be restricted in the size of their DNA due to limited space in the capsid <sup>77</sup> and this has been used to explain the existence of overlapping genes (out of phase, in phase, and antiparallel) <sup>132</sup> often observed in phages <sup>88</sup>. Therefore it seems like a paradox that the phage would carry a gene (*sav*) which is not used. On the other hand, such “gene redundancy” has been observed in other phages as for example the large (170 kbp) genome of the coliphage T4 <sup>82</sup>. Among many other T4 genes, several early expressed proteins used to re-direct the host RNA polymerase (RNAP) from host to phage gene transcription were demonstrated to be non-essential for phage proliferation <sup>113</sup>. However, this gene redundancy is only valid for the conditions under which the redundancy of the gene was tested (normally under optimal laboratory conditions) <sup>87</sup> and redundant pathways has been suggested to be an important factor in the great success of phages as molecular parasites since it allows the phage to adapt to several physiological and environmental conditions <sup>87,113</sup>.

The middle region of the SaV protein is highly conserved among very distantly related phages (infecting gram positive hosts, gram negative hosts and cyanobacteria). This region also contained the majority of the mutations in the phage mutants, which led to either radical amino acid changes or prevented translation, thereby indicating the involvement of a translated SaV protein in the Abi phenotype. A phylogenetic analysis of this conserved region clustered phages belonging to the same group together suggesting that this region of SaV has evolved from a common ancestor (Manuscript II).

Induction of SaV demonstrated a fast working and severe toxic effect in both *L. lactis* and *E. coli* (Manuscript II). Interestingly, a mutated version of SaV with amino acid changes in the conserved region had lost the toxic property indicating that this region contains the active site for both toxicity and interaction with AbiV (Manuscript II + III). It is often observed that early transcribed phage

proteins can have antimicrobial properties when overexpressed in bacterial cells. In many cases this is caused by redirection or inactivation of critical processes in the bacterial cell machinery that facilitates production of phage components<sup>92</sup>. These effects include re-direction of transcription in *E. coli* by interaction with the host RNAP<sup>115</sup> or  $\sigma_{70}$  transcription factor<sup>121</sup> or re-direction of DnaB helicase to favour phage DNA replication<sup>101,120</sup>.

In the present study it was demonstrated that during infection with wild type p2, total RNA and protein synthesis is inhibited (Manuscript III), suggesting that such early re-direction mechanisms also exist in lactococcal phages. Intuitively, this makes sense since the lactococcal phages do not carry their own RNA polymerase and thus are dependent on host factors for both transcription and translation of phage genes. In these situations where the phage has to compete for RNAP and ribosomes with the host, it is often observed that early phage encoded proteins shut-down host RNA and protein synthesis<sup>82,87</sup>. Since the function of these early regulatory proteins is to ease competition for host factors, they are usually not essential under normal laboratory conditions<sup>113</sup>. It can be speculated that SaV due to its early expression and quick toxic effect directed against conserved components in *L. lactis* and *E. coli*, its evolutionary relationship with a wide range of phage proteins, and redundancy in the lytic cycle might be such an early regulatory protein.

### **Interaction of AbiV and SaV**

We demonstrated a direct interaction between the two proteins AbiV and SaV using several different methods. Using gel-filtration, the native form of both proteins was shown to be a dimer (Manuscript I and II), which was confirmed by the SEC-MALS/UV/RI, fluorescence quenching, and cross-linking experiments (Manuscript III). The cross-linking assay suggested that in addition to the interaction between the two homodimers a distinct interaction also existed between monomers of the proteins that reacted to form an AbiV-SaV heterodimer (Manuscript III). This observation certainly demonstrates that AbiV interacts with SaV but the observation of heterodimers might be an artefact caused by the denaturation step in the assay that may have broken the homodimer interactions in AbiV and SaV. The strength of data obtained in the SEC-MALS/UV/RI and fluorescence quenching assays is that the observations are made on native proteins. It is therefore most likely that the AbiV and SaV interaction occurs between two homodimers.

We previously argued that translated AbiV and SaV polypeptides are involved in the Abi phenotype (Manuscript I and II). It is thus possible that the direct AbiV-SaV interaction is mediating the Abi

phenotype for example by activation of the non-toxic AbiV by SaV. Phage induced activation of Abi mechanisms are common in Lactococci<sup>8,26,28,51</sup> and also in the well described phage exclusion systems in *E. coli*<sup>137</sup>. Activation by phage induced transcription of the Abi gene upon infection<sup>26,3,52</sup> is the mechanism when the Abi gene itself is toxic as demonstrated for AbiD1, AbiK, AbiN and AbiO<sup>26</sup>. We showed in Manuscript I that high expression of AbiV has no effects on cellular growth rate and thus that the cellular death caused by the AbiV system must be due to a subsequent reaction with a phage component. This observation supports the hypothesis that the AbiV phenotype is caused by the AbiV-SaV protein interaction.

The interaction sites in the two proteins are not known. However, the mutations in the conserved region of the *sav* gene suggested that this region is involved in the interaction with AbiV (Manuscript II). Furthermore, the mutations also caused the protein to lose the antimicrobial properties (Manuscript II), suggesting a dual function of this region of SaV.

### **Effects on the phage life cycle**

Transcription of phage genes was negatively affected during infection of cells harboring AbiV (AbiV<sup>+</sup> cells). In the early region we observed a 25-40 % decrease of transcription in AbiV<sup>+</sup> cells compared to AbiV<sup>-</sup> cells, whereas the middle and late regions showed a decrease of 50 % and 90 % respectively (Manuscript III). The transcription data suggested continuous mRNA production both in AbiV<sup>+</sup> and AbiV<sup>-</sup> cells throughout the lytic cycle which was supported by an experiment measuring cumulated RNA synthesis through <sup>14</sup>C-uridine incorporation into RNA.

Contrary to RNA synthesis, phage protein synthesis was severely inhibited by AbiV. From the beginning of the phage infection, total protein synthesis rate was diminished and synthesis ceased completely after 15 minutes. According to transcription data from phage sk1 this prevented translation of most middle transcripts (7-10 min after infection) and all late transcripts (15 min after infection)<sup>21</sup>. As observed in Manuscript III, translated SaV was not detectable in the AbiV<sup>+</sup> cells while transcription of genes in the early region (including *sav*) was only minimally inhibited by AbiV. This strongly indicates that the AbiV system targets a component in the cell translation apparatus quickly after infection and that the effects on transcription probably are secondary. Since our data indicates that the AbiV system consists of an AbiV-SaV complex but no SaV translation was observed in the AbiV<sup>+</sup> cells, we suggest that very small amounts of SaV were produced, which reacted with AbiV to cause the AbiV phenotype. However, the western blot method might not have been sensitive enough to detect low levels of SaV expression.

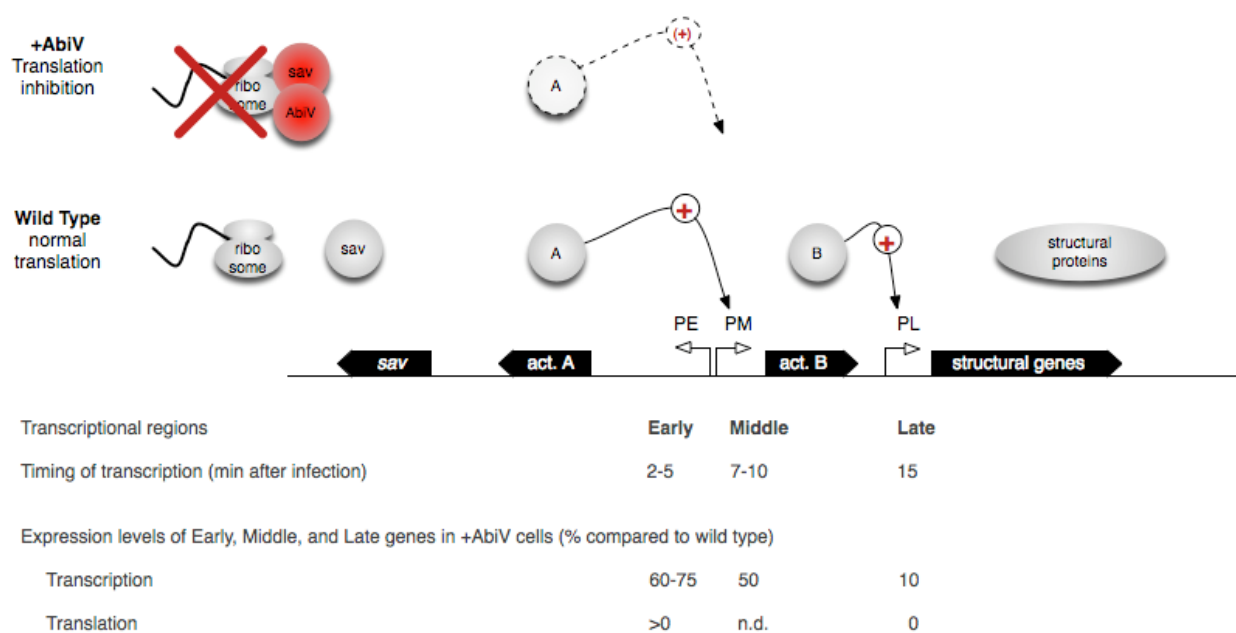


It was not possible to compare our transcription and translation data to other studies since such a time-course experiment with investigation of phage transcription and translation during a phage infection has not been performed with other lactococcal Abi systems.

One way to characterize Abi mechanisms is if they work before or after phage DNA replication<sup>54</sup>. When DNA replication is observed, it is usually in the concatemeric (non-mature) form<sup>3,11,14,51</sup>, which can be caused by arrest of the lytic cycle prior to DNA packaging<sup>20,78</sup> or cell death before the end of the lytic cycle<sup>26,50</sup>. We observed replicated but concatemeric phage DNA when AbiV<sup>+</sup> cells were infected with 936-like phages while no DNA replication was observed when the same host was infected with c2 phages (Manuscript III). Since the same host was used, differences between the two phage types must account for the observed different phenotypes<sup>62</sup>. Similarly, different DNA replication patterns have been observed for other Abi systems when tested against the three phage species 936, c2, and P335<sup>26,40,143</sup>. For example, AbiK allowed DNA replication of phage p2 (936-species) but not ul36 (P335-species)<sup>14</sup> and DNA replication of c2 and 936 phages<sup>117</sup> but not of P335 phages was observed in AbiG<sup>+</sup> cells<sup>143</sup>. Interestingly, the level of AbiV expression was not important for the Abi phenotype against 936-like phages, while protection against c2 infection required high expression of AbiV (Manuscript I) as observed previously<sup>40,117,52,143</sup>. If the Abi phenotype involves the conserved region in SaV (which is common among 936 and c2 phages) it may be speculated that the same mode of action (translation inhibition) is imposed on both phage species. This would imply that the AbiV system must act before translation of the DNA polymerase in c2<sup>17,94</sup>. The DNA polymerase subunits are transcribed early in the lytic cycle from consensus *Lactococcus* promoters in both the 936 phages sk1 and p2 and the c2 phage. However, in c2, the *sav* homologue is transcribed from a very strong promoter compared to the promoter in front of the DNA polymerase<sup>93</sup>. This strong early promoter was suggested to be among the strongest lactococcal promoters identified<sup>93</sup> and it is thus possible that transcription of the *sav* homologue in c2 from such a strong promoter could activate AbiV and thus the Abi phenotype before translation of the DNA polymerase. This promoter type was not observed in the analyzed 936 phages.

### **A model for the molecular mode of action (MOA) of the AbiV system**

Based on the data in Manuscripts I-III, the following hypothesis on the MOA of the AbiV system against 936-like phages was constructed (Fig. 6). It should be noted that several parts of the hypothesis are still speculations and further studies are needed to clarify the exact molecular MOA of the AbiV system.



**Fig. 6.** Model of the MOA of the AbiV system against 936 phages. Black boxes symbolize phage genes where act.A and act.B are putative phage encoded activators encoding proteins A and B, respectively. PE, PM, and PL are early, middle, and late promoters respectively. In wild type cells translation occurs normally, ActivatorA induces middle transcription where ActivatorB is expressed, which in turn activates the late promoter for transcription of structural genes.

In AbiV<sup>+</sup> cells, SaV activates AbiV and translation is shut down. ActivatorA is therefore only produced in small amounts, ActivatorB is not produced and the lytic cycle is arrested. Transcription and translation levels are presented as percentage in AbiV<sup>+</sup> cells compared to wild type.

Shortly after infection, genes in the early region of the phage are transcribed from several lactococcal consensus promoters as described for phage sk1<sup>22</sup> and translation of these transcripts starts. Among the early transcripts is the phage DNA polymerase<sup>17</sup> and presumably also phage proteins that induce the observed arrest of host RNA and protein synthesis (Manuscript III)<sup>92</sup>. SaV is translated quickly after infection, which triggers interaction with AbiV and subsequent shut-down of the translational machinery of the cell (Fig. 6). Presumably, SaV is only needed in small amounts to induce the Abi phenotype. This causes early arrest of translation, including the translation of SaV, which is therefore only produced in sub-detectable amounts (Manuscript III).

The early inhibition of translation probably also prevents expression of a putative phage encoded activator, which like in phage sk1 is needed for transcription of the middle genes<sup>8,22,86</sup> thereby causing the 50 % decrease in middle transcripts (Manuscript III).

As in phage TP901-1<sup>18</sup>, the middle operon of phage sk1<sup>21</sup> and p2 probably contains an activator of late gene transcription that induces transcription just downstream of the activator gene. In AbiV<sup>+</sup> cells such an activator would be translated at very low levels due to the combined effect of inhibited transcription of the middle region and the translation inhibition by the Abi mechanism. Altogether

this could explain the very limited transcription of late genes (Manuscript III and Fig. 6). Interestingly, a putative RNA binding site was observed in AbiV using different predictor software<sup>144,145</sup>. Since the native AbiV homodimer is not harmful to the cell (Manuscript I), it could be speculated that this RNA binding site is only exposed due to a conformational change upon interaction with the SaV protein. RNA binding by the activated AbiV-SaV complex could thus be part of the MOA of the system, though it is unlikely that the RNA binding leads to RNase activity (as observed with AbiB<sup>122</sup>). It is interesting to note the similarities between the MOA of AbiV and the two well characterized *E. coli* phage exclusion systems Lit and PrrC<sup>137</sup>. For all three systems it was observed that the abortive infection enzyme is constitutively expressed in an inactive form which is activated by a small phage encoded peptide upon infection. The activated protein subsequently functions by cleaving conserved and essential components of the cell translation apparatus thereby killing the cell and preventing phage proliferation.

### **Industrial applications of the AbiV system**

The dairy industry has worked more than 70 years to protect their production strains against phages and though significant progress has been made, phages remain a significant problem for the dairy industry. Thus it has been estimated that between 0.1 % and 10 % of all milk fermentations are negatively affected by phage attacks thereby causing substantial economical losses to the industry<sup>109</sup>. Phages are ubiquitous in the environment and many phages are still viable after pasteurization of the raw milk used in fermentation<sup>25</sup>. Phage control is therefore focused on several phases of the fermentation process. Measures such as proper hygiene, disinfection and efficient air-filtration are important in combination with rotation of the mixed starter cultures to control phage infections<sup>29,109</sup>. Also natural phage resistance mechanisms, which are often encoded on conjugative plasmids have been used extensively and systematically since the discovery of plasmids harboring different phage resistance mechanisms<sup>135</sup>.

The extensive use in a relatively small number of industrial starter strains<sup>33</sup> has favored the emergence of phage mutants that are insensitive to the applied anti-phage barriers<sup>34,54,107</sup> thereby driving the need in the dairy industry for constantly seeking new anti-phage strategies. Different types of engineered phage resistance mechanisms have been developed<sup>42,43,104,72,97,103,31</sup>, but limited efficiency<sup>81</sup> and more importantly legislative obstacles<sup>54,107</sup> as well as consumer skepticism<sup>33,54,153</sup> of genetically modified organisms (GMO) make this approach less attractive. Therefore, isolation and conjugal transfer of novel anti-phage barriers remain an important strategy for the dairy

industry for protection against phages<sup>109</sup>. The efficiency of protection against phage attack is measured as efficiency of plaquing (EOP) and varies from  $10^{-1}$  to  $<10^{-8}$  for the different isolated Abi mechanisms<sup>32,51</sup> (Introduction, Table 2). The EOP of AbiV is ca.  $10^{-4}$  and the system is thus characterized as a medium strength Abi system<sup>107</sup>. AbiV is a novel abi mechanism, i.e. similarity to other amino acid sequences or MOA with an interacting phage component of other Abi mechanisms was not found. Furthermore, since AbiV was demonstrated to work in both subspecies of *L. lactis* (*lactis* and *cremoris*) it is suitable to work either as a single anti-phage barrier or in combination with other phage resistance mechanisms.

We demonstrated in Manuscript IV that *L. lactis* can mutate to spontaneously express AbiV and thus confer phage resistance to the host. This can be of great industrial value as the AbiV phenotype can be applied to strains without the use of genetic modification. However, most R/M and Abi systems are encoded on plasmids<sup>11,14,26,50,51,60,73,117,150</sup>, many of which are conjugative and for the chromosomally encoded phage resistance mechanisms as AbiV, the industrial application is dependent on the ability to transfer the resistance gene(s) to industrial production strains<sup>2,29,66,107,116,130,140</sup>. We demonstrated that *abiV* can be transferred between lactococcal strains via chromosomal conjugation (Manuscript IV). Furthermore it can be speculated that *abiV* due to its location in the *L. lactis* MG1363 “integration hot spot” region where it is flanked by two putative transposases (*lin* and *trans*) can be mobilized onto a conjugative plasmid.

## PERSPECTIVES AND OUTLOOK

The results presented in this thesis have provided a detailed description of a novel lactococcal phage abortive infection system and its mode of action. This includes isolation and characterization of both the bacterial protein AbiV and the phage protein SaV that interact directly to cause the Abi phenotype. Moreover, the mode of action was revealed to inhibit protein synthesis early in the lytic cycle of 936-phages. A model explaining the possible molecular interactions of the AbiV system was proposed and the results altogether provide one of the most comprehensive descriptions of a lactococcal Abi system.

Due to their economical value, lactococcal Abi systems are among the best investigated bacterial Abi mechanisms<sup>81</sup>. Still, for most of the isolated lactococcal Abi systems, the mode of action is unknown and they are often only characterized according to their action early (before phage DNA replication) or late (after phage DNA replication) in the lytic cycle<sup>62,81</sup>. In the few cases where the mode of action has been revealed, the interaction of lactococcal Abi mechanisms with the lytic

cycle of phages has proven to be complex<sup>13,14,26,40,52,55</sup>. This is supported by reports of a single Abi mechanism using different modes of action against the three main lactococcal phage species.

The characterization of lytic lactococcal phages including the function of most of their early transcribed proteins and the molecular processes during the lytic cycle is virtually unknown. For some *E. coli* phages (e.g. T4), the lytic cycle is described in much greater detail and has revealed an efficient molecular parasite that adapts to environmental changes through a complex network of interactions<sup>82,87,113</sup>. Though the genomes of lactococcal phages are much smaller (13% and 17% of the T4 genome for the phages c2 and sk1, respectively) it is likely that early proteins of lactococcal phages are involved in interaction networks with both phage and host factors and that Abi mechanisms through a variety of phage targets exhibit complex mode of actions that differ according to the infecting phage.

Thorough investigation of the different Abi mechanisms and the interaction with genes and gene products in phages from all three main species (936, c2, and P335) is thus necessary to obtain the knowledge that can help designing future starter cultures with improved phage resistance properties.

Obtaining the three-dimensional structure of the Abi protein, the interacting phage protein as well as the complex of the two (when protein-protein interaction occurs) will provide a powerful tool in determining the protein functions, their active sites and relationship (functional and or evolutionary) to other proteins<sup>128,138,139,149</sup>. Ongoing work in collaborating groups is focused on determining the structure of AbiV, SaV and the AbiV-SaV complex in an attempt to elucidate the exact molecular function of the AbiV system. It would be of great interest to reveal the specific mechanism of which the complex inhibits protein synthesis by combining the structure data (if obtained) with RNA binding and in vitro translation assays to test if the complex binds RNA and maybe prevents translation by steric hindering of the ribosome access to the mRNA.

If 3D structure information of SaV is obtained, it might also prove to be valuable for determination of the host target of SaV that causes the strong antimicrobial effect when the protein is expressed in *E. coli* or *L. lactis*. The identification of the SaV host target might also be determined using affinity chromatography as described by Liu *et al.*<sup>92</sup> or by sequence analysis of SaV resistant bacterial mutants. The possibility of SaV being a novel antimicrobial molecule makes it very interesting to pursue the investigation of this protein.

Finally, from an industrial point of view, it would be interesting to mobilize *abiV* from the chromosome of *L. lactis* MG1363 onto a conjugal plasmid for use in industrial production strains.



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